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Original Article

Investigation of Antioxidant Markers in Diabetic Patients

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Abstract

Hyperglycemia is a characteristic of diabetes mellitus, one of the most common metabolic illnesses in the world, and is caused by either reduced insulin secretion or insulin resistance. Diabetes mellitus in adults has been on the rise in recent decades, and it is now the world's fifth-biggest cause of mortality. Diabetes mellitus will affect 592 million people worldwide by 2035, according to the International Diabetes Mellitus Federation, and diabetic complications are divided into two groups of acute and chronic types. Diabetic ketoacidosis, nonketotic hyperosmolar coma, and hypoglycemia are some examples of acute problems, whereas chronic complications include injuries to the small vessels (microvascular issues) and large blood arteries (macrovascular complications). Diabetic neuropathy, retinopathy, and nephropathy all have microvascular consequences, and on the other hand, macrovascular problems have a role in the etiology of cardiovascular disorders such as coronary, cerebrovascular, and peripheral artery diseases. This study aimed to estimate some antioxidant markers, including total protein, albumin, globulin, albumin/globulin ratio (AGR), free amino, free amino/total protein, thiol, thiol/total protein, carbonyl, as well as carbonyl/total protein levels in the plasma of diabetic complications compared to healthy subjects, and investigate the correlations between them. The present study included 120 plasma samples divided into 80 samples as patients with diabetic complications; 26, 26, and 28 samples had diabetic kidney disease, diabetic retinopathy, and diabetic neuropathy, respectively, with the age range between 20-60. Moreover, a total of 40 healthy subjects were included in the study as the control group with the same age ranges. The results showed that there was not any significant difference in carbonyl; however, significant differences were recorded in the total protein, albumin, globulin, AGR, free amino, free amino/total protein, thiol and thiol/total protein, as well as carbonyl/total protein levels in all studied groups. The correlation outcomes indicated that there were significant positive relationships between total protein-globulin, AGR-albumin, and free amine-albumin. In contrast, significant negative correlations were recorded between total protein-AGR and AGR-globulin in diabetic complications. Finally, it was concluded that oxidation markers might play a role in monitoring diabetic complications.

Keywords: Antioxidant markers, Diabetic complications, Total protein

1. Introduction

Approximately 50% of the patients with diabetes mellitus (DM) are expected to be unaware of their illness, making them more prone to develop diabetic complications (1). Diabetes hazards are common in people with diabetes type 1 or 2; however, they are frequently associated with high morbidity and mortality rates. The chronic complications of diabetes are divided into two categories of "microvascular" such as neuropathy, nephropathy, as well as retinopathy, and "macrovascular," including neuropathy, nephropathy, retinopathy, cardiovascular disease, stroke, coronary artery disease, diabetic foot syndrome, peripheral artery disease, and inflammation. On the other hand, chronic complications of diabetes are the significant causes of lower limb amputation (2, 3). Finally, some other problems related to diabetes are dental diseases, reduced disease susceptibility, and delivery issues in

women with gestational diabetes (2). During the last decade, the use of plasma protein in diagnosing numerous diseases and the provision of pathophysiological information has expanded dramatically (3). In general, it has been noted that the variations in plasma protein levels can be caused by any change in catabolism rate, anabolism rate, and distribution volume (4). However, there is a scarcity of knowledge about lycopene-induced albumin modifications (resulting from changes in hemoglobin metabolism) (5). High globulin levels have also been linked to an increased risk of diabetes (6). Human serum albumin (HAS) is the most abundant extracellular protein, involved in various tasks such as molecular transport, colloidal osmotic pressure management, and serum redox status buffering (7).

A free cysteine residue at position 34 from the N-terminus of HAS is responsible for the buffering action on serum redox status (Cys-34). Cys-34 of HSA deoxidizes other chemicals depending on the level of oxidative stress in the environment, and it is also oxidized (a sacrificial antioxidant). HSA is a mixture of human mercaptalbumin (HMA), which is considered to have a disulfide link that can be reversibly oxidized by cysteine, and human nonmercaptalbumin (HNA), which is strongly oxidized and becomes a sulfenic (-SOH), sulfinic (-SO2H), or sulfonic (-SO3H) species (8). The albumin/globulin ratio (AGR) is a test that is used to predict liver and renal disease (9) and could reveal information regarding a person's health concerns (10) and typical factors for determining the rank of systemic inflammation; in addition, this metric is simple and inexpensive to measure. Hepatic cells are the primary producers of albumin and globulin; low serum albumin levels can be caused by hepatic failure, malnutrition, or systemic inflammation. On the other hand, infectious illness and (or) systemic inflammation cause serum globulin levels to rise. As a result, the negative relationship between these two measures in terms of systemic inflammation status can significantly affect the AGR in patients with cancer (11). Non-specific indicators of protein modification include the oxidation of the thiol group of protein-bound cysteine residues; many oxidants can cause this reaction to occur (12). Thiols are key components of the whole redox buffer system and protect the body from oxidative stress. In addition, antioxidant defense mechanisms are complicated and multifactorial (13). Thiols with a sulfhydryl (-SH) group, which are the principal target of reactive oxygen species, are oxidized by oxidant molecules to produce reversible disulfide HSA, accounting for approximately 80% of the total free thiol content in plasma and the status of Cys-34. Because of the short half-life (20 days) of the albumin molecule, the redox status of HSA is suggestive of the degree of short-term systemic oxidative stress (7). In addition to oxidative stress, chronic renal disease, diabetic retinopathy, and neuropathy have all been linked to an increase in carbonyl production due to long-term oxidative damage to lipids, carbohydrates, and proteins (14). Protein carbonylation is thought to reflect carbonyl modification of amino acid residues, which is made up of amino acids such as lysine, arginine, proline, and threonine, and it is the most general and extensively used biomarker of the state of long-term carbonyl overload or "carbonyl stress." Protein carbonyl concentration is the most general biomarker of protein oxidation, and to a large extent, the most frequently employed marker (15).

Carbonylated proteins have several advantages over other oxidation products because of their relative early production and stability. Because of secondary reactions, multiple radicals, excited state species, and singlet oxygen produce protein carbonyls; dicarbonyls have a well-established involvement in synthesizing these chemicals (16). This study aimed to estimate some levels of antioxidant markers, including total protein TP, albumin, globulin, AGR, free amino, free amino/TP, thiol, thiol/TP, carbonyl, and carbonyl/TP, in the plasma of patients with diabetic complications compared to healthy subjects, and then study the correlation between them in the case group.

2. Materials and Methods

2.1. Study Design, Subjects, and Samples Collection

The present study included 120 plasma samples divided into 80 samples as patients with diabetic complications; 26, 26, and 28 samples had diabetic kidney disease, diabetic retinopathy, and diabetic neuropathy, respectively, with the age range between 20-60, a total of 40 healthy 20- to 60-year-old subjects as the control group, and people who referred to Kirkuk general hospital in Kirkuk, Iraq, from October 2020 to March 2021 and diagnosed by specialist doctors. A total of 2–3 ml of blood was taken by vein puncture in glass tubes within an EDTA tube using a disposable syringe. The tubes were centrifuged for 10 min at 1500g, the buffy covering was removed after the plasma was separated from the cells, and then plasma samples were kept at 20°C until used.

2.2. Biochemical Assays

Albumin level was determined using the BCG method (17), the premise of which involves binding albumin with BCG dye to produce a blue-green color that can be detected at 630 nm, with the increase in color proportional to the amount of available albumin. A technique proposed by Lowry and Hunter (18) was used to determine total serum protein levels, and BSA was utilized as a control protein. The determination of free amino group spectrophotometric was carried out using the method proposed by Zaia, Barreto (19). The levels of thiol groups were estimated according to Ellman (20), which has been modified by Riddles, Blakeley (21) using the equation $A = Ɛ. C. l$, where: $Ɛ=14{,}100\ M^{-1}\ cm^{-1}$. The protein carbonyl content was assayed according to the method presented by Levine, Garland (22) using the equation $A = Ɛ. C. I$, where: $Ɛ=22{,}000\ M^{-1}\ cm^{-1}$.

2.3. Statistical Analysis

Statistical analysis was performed using GraphPad Prism v8.0 (GraphPad Software, San Diego, CA, USA), and mean standard error (SE) was used to express the results. The comparison of mean±SE was performed using the ANOVA test, and statistical significance was defined as $P\leq0.05$ and the correlation between the parameters.

3. Results

Table 1 represents the levels of TP, albumin, globulin, AGR, thiol, thiol/TP, free amine, free amine/TP, carbonyl, and carbonyl/TP) as mean±SE of plasma patients with DM complications in comparison with healthy subjects.

The results indicated that there was a non-significant decrease ($P\geq0.05$) in carbonyl levels, a significant decrease ($P\leq0.05$) in TP, albumin, globulin, as well as AGR levels, and a significant increase ($P\leq0.05$) in free amine, free amine/TP, carbonyl/TP, Thiol, as well as Thiol/TP levels in case groups compared to healthy subjects. Table 2 represents the levels of each parameter as mean±SE of plasma patients with DM complications divided to diabetic (kidney, retinopathy, and neuropathy) compared to healthy subjects.

Table 1. Levels of some biochemical parameters as mean±SE for all studied groups

Parameters	Healthy subjects (n=40) mean±SE	Diabetic complication (n=80) mean±SE	P-value
TP (gm/dl)	7.102±0.5495	4.107±0.2906	≤0.05
Globulin (gm/dl)	2.778±0.5834	1.917±0.4184	≤0.05
Albumin (gm/dl)	5.3735±7.180	2.183±0.2924	≤0.05
AGR	2.138±3.002	1.204±0.3629	≤0.05
Thiol (µmol/L)	18.52±2.960	32.60±10.50	≤0.05
Thiol/TP (µmol/gm)	26.17±4.343	80.18±28.05	≤0.05
Free amine (mmol/L)	34.62±10.82	54.07±17.65	≤0.05
Free amine/TP (mmol/gm)	49.52±17.54	131.9±43.92	≤0.05
Carbonyl (nmol/ml)	80.36±9.589	78.86±10.44	≥0.05
Carbonyl/TP (nmol/gm)	0.1137±0.01567	0.1932±0.03018	≤0.05

Table 2. Levels of some biochemical parameters as mean±SE for case groups as diabetic (kidney, retinopathy, and neuropathy) compared to healthy subjects

Parameters	Healthy subjects (n=40) mean±SE	Diabetic kidney (n=26) mean±SE	Diabetic retinopathy (n=26) mean±SE	Diabetic neuropathy (n=28) mean±SE
TP (gm/dl)	7.082±0.5315[a]	4.040±0.2978[b]	4.533±0.3809[c]	4.203± 0.638[d]
Globulin (gm/dl)	2.778±0.5834[a]	2.037± 0.4362[b]	1.776±0.499[c]	1.955±0.274[d]
Albumin (gm/dl)	6.184±8.675[a]	2.209±0.2975[b]	2.409±0.465[c]	2.118±0.240[d]
AGR	2.138±3.002[a]	1.247±0.493[b]	1.442±0.366[c]	1.120±0.275[d]
Thiol (µmol/L)	18.52±2.96[a]	33.43±8.01[b]	38.16±12.82[c]	27.20±6.66[d]
Thiol/TP (µmol/gm)	26.17±4.34[a]	83.18±20.94[b]	92.56±38.22[c]	74.25±22.51[d]
Free amine (mmol/L)	34.62±10.82[a]	68.77±19.11[b]	54.00 ±16.34[c]	44.81±10.56[d]
Free amine/TP (mmol/gm)	49.52±17.54[a]	171.9±48.54[b]	128.3±38.10[c]	109.8±25.31[d]
Carbonyl (nmol/ml)	80.36±9.589[a]	81.58±5.501[a]	80.16±8.644[a]	75.68 ±13.52[a]
Carbonyl/TP (nmol/gm)	0.114±0.016[a]	0.1990±0.022[b]	0.1981±0.025[c]	0.1856±0.037[d]

[a,b,c,d] Different letters are referred to as significant differences, while similar letters denote non-significant differences between the compared groups ($P \leq 0.05$ significant; $P \geq 0.05$ non-significant); AGR: Albumin/Globulin ratio

The outcomes presented in table 2 indicate that there are significant differences ($P \leq 0.05$) between all parameters and non-significant differences ($P \geq 0.05$) in carbonyl levels in diabetic patient groups (kidney, retinopathy, and neuropathy) compared to healthy subjects.

Table 3 represents the Correlation coefficient between all studied parameters in case groups.

Table 3. Correlation coefficient between the studied parameters in patients with diabetic complications.

Variables	r/P
TP-Thiol	-0.01310/0.9143
TP-Free amine	0.08882/0.4647
TP-Carbonyl	-0.06481/0.5940
Carbonyl-Thiol	-0.01232/0.9194
Carbonyl-free amine	-0.03393/0.7803
Thiol-Free amine	0.2014/0.0946
TP-Albumin	0.1993/0.0981
TP-Globulin	0.6558/0.0001
TP-Albumin\Globulin ratio	-0.4308/0.0002
AGR-Albumin	0.4990/0.0001
Globulin-Albumin	-0.04258/0.7264
Thiol-Albumin	-0.03129/0.7971
Free amine-Albumin	0.2881/0.0156
Carbonyl-Albumin	0.08012/0.5097
Carbonyl-Globulin	-0.1420/0.2408
Free amine-Globulin	-0.05396/0.6573
Thiol-Globulin	-0.09899/0.4151
AGR-Globulin	-0.8337/0.0001
AGR-Thiol	0.07605/0.5315
AGR-Free amine	0.2113/0.0791
AGR-Carbonyl	0.1939/0.1077

*$P \leq 0.05$ significant; r/P: Correlation coeffecient/Significance; AGR: Albumin/Globulin

The outcomes of correlation indicated a significant positive relationship between TP-globulin, AGR-albumin, and free amine-albumin. Moreover, there was a significant negative correlation between TP-AGR and AGR-globulin in patients with diabetic complications.

4. Discussion

The proteins are primary targets for oxidative damage because of their abundance and quick reaction rates with an extensive range of radicals and excited state species. In the case that proteins are exposed to oxidants, the parent amino acid residue is lost, unstable intermediates are formed, and stable products are produced; each of these events can be utilized more or less to quantify protein damage. By assessing the loss of individual amino acids, total free amino group analysis can provide information on the nature of oxidation events occurring in poorly understood systems (12). The results of TP, albumin, globulin, and AGR levels indicated a significant ($P \leq 0.05$) decrease in the case group compared to healthy subjects. These results were in line with Abd (23) and Abd Alkader (24); they had also found a decrease in the levels of TP, albumin, globulin, and AGR in β-thalassemia, DM, as

well as diabetic nephropathy patients. The results were contrary to the findings of Azeez (25) and Noah (26); they had found an increase in the TP levels of patients with breast cancer and DM, respectively. The possible reason for the decrease in the parameters mentioned above is the excessive sugar and the natural filtration of the kidneys, which leads to the accumulation of toxic wastes. Moreover, large amounts of TP, albumin, and globulin are lost with urine, which may lead to a lower amount of albumin in the case group. Albumin and globulin act as transport proteins and biomarkers in the case of diabetes complications (27). The reduction in TP levels can also be explained as the lack of natural feedback inhibition of gluconeogenesis in the liver that causes an increase in the breakdown of lipids and proteins, as well as the conversion of glucogenic amino acids to glucose which leads to an increase in glucose levels; in addition, hemodilution could also be a cause of this reduction. Increased urine in diabetic individuals occurs due to high glucose levels; this rise causes the cell to pump water (hydration) into the bloodstream, causing increased urinary and glucose dilution (28). The AGR was also measured, which can give some clues about problems in the body (10), and the AGR representative metrics were also assessed, the measurement of which are simple and inexpensive and they are useful to rank systemic inflammation. Hepatic cells are the primary producers of albumin and globulin and low serum albumin levels can be caused by hepatic failure, malnutrition, or systemic inflammation (11). The results of free amino, free amino/TP, thiol, thiol/TP, and carbonyl/TP levels indicated a significant ($P \leq 0.05$) increase in the case group compared to healthy subjects. These results were in line with Abd (23), Abd Alkader (24), and Azeez (25), they had also found that there were increases in the free amino as well as free amino/TP levels in patients with β-thalassemia, DM, diabetic nephropathy, and breast cancer. Moreover, these results were contrary to the findings of Trifunovic-Macedoljan, Pantelić (29) and Noah (26); they had found that there was a decrease in the free amine group level in DM patients. The reason for the increased level of amino groups might be due to the elevated circulating levels of the branched-chain amino acid (BCAAs) and central nervous system (Acyl. CNsO) associated with fatty acids in DM patients (30). The increased levels of BCAAs may be due to the catabolism of enhanced BCAAs leading to an increase in plasma levels of propionyl carnitine (C3) and iso-valeryl carnitine (C5) in DM patients (31). Many studies suggested using the "free amino and free amino/TP" level as markers in the pathological states (32). The results of carbonyl/TP, thiol, and thiol/TP levels revealed a significant increase ($P \leq 0.05$) in patients with diabetic complications; these results were in line with Abd (23), Abd Alkader (24), Noah (26), and Azeez (25); they has also found an increase in carbonyl/TP levels in patients with β-thalassemia, diabetic nephropathy, DM and breast cancer. The increase in carbonyl/TP levels might cause the patients' aerophilic stress and inflammation (33) or oxidative stress, which occurs in various human diseases and inflammation as an imbalance toward the pro-oxidant side of the pro-oxidant/antioxidant equilibrium (33). The results of the present study revealed a non-significant decrease ($P \geq 0.05$) in carbonyl levels in patients with diabetic complications. Moreover, the decrease in the carbonyl level might be due to reactive oxygen species (ROS) that can target proteins and oxidize the amino acid side of protein bonds, resulting in extra carbonyl groups (protein carbonyl) (34). The results of carbonyl groups were in contrast to the findings of Abd (23), Abd Alkader (24), and Noah (26); they had found an increase in carbonyl levels in patients with β-thalassemia, diabetic nephropathy, and DM. These results were in line with the findings of Abd (23), Abd Alkader (24), and Noah (26) regarding thiol and thiol/TP levels. The increase in thiol levels observed in this study could be related to antioxidant protection in these conditions, where the patients' population is at high risk of tissue oxidant injury due to reduced plasma antioxidant defense (35). Thiol group is

a strong nucleophile, as its physiological pH values are stronger compared to that of "amine and carbonyl" groups with Lys and Arg side chains (36). The significance of the sulfhydryl group (SH) and D-glucose reactions in protein cross-linking has recently been discovered by Zeng and Davies (37) and 2006Zeng and Davies (38). Moreover, Westwood et al. proposed that the initial product of a reaction between a thiol group and D-glucose can be used as a target for the next reaction with an amine group, and vice versa, the initial product of an amine group and D-glucose can be used as a target for this group (39).

5. Conclusion

The results of the present study indicated that the oxidative proteins markers might have a role in diabetic complications compared to healthy subjects, which might actively contribute to the progression of the disease. The mentioned findings may support an association between these oxidation proteins and diabetic complications. The stronger response was observed in plasma (TP, albumin, globin, albumin/globin ratio, thiol, thiol/TP, free amine, free amine/TP, and carbonyl/TP) of patients with the change in the level of proteins suggesting that contents of these oxidative protein markers may be useful in evaluating the disease pathogenesis. Positive correlations between TP-globulin, AGR-albumin, and free amine-albumin, as well as negative correlations between TP-AGR and AGR-globulin in diabetic complications, may be important for evaluation and diagnosis of diabetic complications of patients.

Authors' Contribution

Study concept and design: M. A. K.
Acquisition of data: M. A. K.
Analysis and interpretation of data: I. G. Z.
Drafting of the manuscript: I. G. Z.
Critical revision of the manuscript for important intellectual content: M. A. K. and I. G. Z.
Statistical analysis: M. A. K.
Administrative, technical, and material support: M. A. K. and I. G. Z.

Ethics

All studies were performed in compliance with the rules of humane treatment of University of Kirkuk, Kirkuk, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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