

Estimation and Isolation of Ceruloplasmin and Some Biochemical Indicators in Diabetes Mellitus Type II Patients Compared to Healthy Controls in Kirkuk Province, Iraq

Karam Yahya Noah, Farah Khalaf Hmood, Israa Ghassan Zainal

Department of Chemistry, College of Science, University of Kirkuk, Kirkuk, Iraq

Abstract

Background: The pathogenesis of diabetes mellitus (DM) was affected by oxidative stress. Many inflammatory markers having antioxidant property, among them ceruloplasmin (CP), which is the appropriate candidate to recognize the general insulin resistance in patients suffering from DM- Type II. **Objectives:** This study aimed to estimate some biochemical parameters (that considered markers of oxidative stress) in patients with DM type II (DM-II) compared to healthy controls and study the correlation between them. **Materials and Methods:** This study included 75 samples of blood serum divided into (50) samples (26 males and 24 females) as patients with DM-II. The control group included 25 healthy controls (15 males and 10 females). Ceruloplasmin (CP) was isolated from human serum using 60% ammonium sulfate precipitation, then estimated the activity and specific activity of enzyme. The isolated enzyme was characterized by 10% polyacrylamide gel electrophoresis. Some biochemical indicators were also estimated. These included random blood sugar (RBS), total protein, thiol, free amino, and carbonyl. **Results:** The electrophoresis results for both protein and glycoprotein stain indicated that the bands of CP in patients group have more intensity than the bands of healthy controls. There were nonsignificant increase in total protein, thiol/protein, carbonyl and carbonyl/protein levels, nonsignificant decrease in the free amino and free amino/protein levels, and significant increase in the RBS and thiol levels in patients group compared to healthy controls. The correlation results indicated that there were significant positive correlation between CP and RBS with $r = 0.306$. **Conclusion:** The findings may support an association between oxidation proteins and DM-II. The stronger response observed in serum CP and thiol from patients with the change in the concentration of proteins which suggest that these oxidative proteins markers contents may be useful in evaluating the progression of DM-II and in elucidating the mechanisms of disease pathogenesis.

Keywords: Ceruloplasmin, diabetes mellitus type II, oxidative stress

INTRODUCTION

Diabetes mellitus type II (DM-II) could be a persistent modern illness, normally defined as a hyperglycemia and dyslipidemia that later ends in vessel complications which affecting kidney, eye, and nervous system also, could impair aldohexose metabolic pathways and increase auto-oxidative glycosylation and free radical production.^[1] Rise in blood glucose levels induce oxidative stress and decrease antioxidant defenses, thus leading to increase free radical formation, which can react with “proteins or lipids” to give rise to oxidative damage.^[2] DM linked to enhancing the flux of reactive oxygen species (ROS) within the living things which associate with the cellular redox system that ends up in a loss of reducing capability, with repercussions on enzymes and antioxidant defense system.

The attack by ROS against proteins modifies amino acid residues generating carbonyl moieties, which can be used as a measure of protein damage.^[3] ROS are continually made as a consequence of normal physiological metabolism by cellular antioxidant defense mechanisms within everyday physiological situations. Normally, the manufacturing and neutralization of ROS are balanced with antioxidants in living systems and

Address for correspondence: Prof. Israa Ghassan Zainal,
Department of Chemistry, College of Science,
University of Kirkuk, Kirkuk, Iraq.
E-mail: israaz@uokirkuk.edu.iq

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did not cause any oxidative harm. The imponderables among those pro-oxidants and antioxidants within the living organism system brings to cellular disruption and damage. These ROS used as oxidative stress significances each *in vivo* and *in vitro* mensuration.^[4]

Many studies confirmed that the rise in oxidative stress is related to insulin resistance pathogenesis by means of insulin indicators suppression.^[5] The oxidative damage provoked by using reactive free radicals which has been established to play a vast position in aging, diabetes and numerous pathological events. Defense systems against oxidative attacks are sometimes ready to stop most reactive species created throughout physiological and pathological metabolisms; but, the imbalance among manufacturing and scavenging of free radicals, due to an increase in oxidative flux or a decrease in the antioxidant capability, is responsible for cell and tissue harm in a number of acute and persistent illness, such as DM.^[3]

Among various inflammatory markers, ceruloplasmin (CP), a copper-carrying metalloenzyme, acts as an antioxidant through its ferroxidase activity, is the appropriate candidate to recognize the general insulin resistance in patient sufferers from DM-II. In conditions of elevated aerophilic stress, CP might act as a pro-oxidant by donating of free copper ions, that motivates ROS forming, the CP level reflects acute and chronic inflammation in an organism.^[1] CP may feature additionally as an antioxidant in two ways: Free copper and iron ions a powerful catalysts of free radical harm, by means that of bound copper CP prohibit free copper ions from catalyzing oxidative harm. It's been implied that enormous CP inactivation can also occur and free copper ions can be launched in the course of publicity to oxidative stress, Therefore, broken the activity of CP could cause the augmentation of free radical-mediated harm to different macromolecules upon exposure to aerophilic stress.^[6]

Thiols, also known as inflammatory markers,^[7] are a class of natural compounds that contain a sulfhydryl group sulfur and a hydrogen atom (–SH) attached to a carbon atom. Thiols will endure oxidation reaction via oxidants and type di-sulfide (RSSR) bonds (which could be a covalent bond). This linkage is likewise noted as S-S bond or di-sulfide bridge. Under conditions of aerobic stress, the oxidation of cysteine residues will result in the reversible formation of mixed di-sulfides bond between macromolecule thiol groups. The fashioned di-sulfide bonds will once more be reduced to thiol groups; so, dynamic thiol di-sulfide physiological state is maintained.^[8]

In hyperglycemia, free amino teams of proteins react slowly with the carbonyl teams of reducing sugars, like aldohexose, to yield a Schiff-base intermediate (Maillard reaction). These Schiff base intermediates bear Amadori rearrangement to stable ketoamine derivative (fructose amine). Carbonyl stress could result from a rise in substrate stress and/or a decrease in the potency of detoxification of carbonyl compounds. It seems that generalized aerophilic stress in DM does not occur. Possibly, exist a neighbor-hood aerobic stress, which can be a vital

modulator for the event of DM complications.^[9] The objective of the present study was to estimate some biochemical parameters in patients with DM-II compared to healthy controls and study the correlation between them.

MATERIALS AND METHODS

Study design and subjects

Seventy-five individuals were chosen in this study; they were divided to: (50) samples consisting of 26 males and 24 females as patients with DM-II, age ranges between (30 and 60) years. Those patients visited K1 hospital/Kirkuk city/Iraq, from December 2018 to May 2019. Ethical approval was given by the Institution, Ethical Committee of K1 Hospital. All patients were subjected to a personal interview using specially designed questionnaire format of full history with detailed information and healthy individuals as control group included (25) participants (15 males and 10 females) with the same age ranges as patients. Any case may interfere with this study such as “hypertension, anemia, and liver diseases” were discarded.

Blood collection

Using a disposable syringe, 2–3 ml of blood was collected by venipuncture in glass tubes within Gel Tube For separated. The tubes were left at room temperature for 15 min then centrifuged at 1500×g for 10 min. The serum was separated from the cells and buffy coat removed. Blood samples were stored at –20°C until used.

Isolation and characterization of ceruloplasmin

Human serum CP were isolated from serum by precipitation with (60%) ammonium sulfate method,^[10] and dialyzed at 4°C against the acetate buffer (0.4M, pH = 5.2) overnight with changing buffer each 8 h, then polyacrylamide gel electrophoresis was performed using 10% separating gel. The gel was cut to three pieces: the first piece was stained using staining protein method with Coomassie Brilliant Blue G-250 by a standard method.^[11] The second piece is used to determine CP activity using p-phenylene di amine (PPD) as a substrate,^[12] and the third piece of the gel was stained with Ponceau S stain (glycoprotein stain) by Leach *et al.*^[13] using bovine serum albumin (BSA) as a standard protein.

Biochemical assays

CP activity was estimated by the method of Al-Helaly and Tareq^[14] using PPD as a substrate. The activity of CP is calculated according to the following equation: CP activity (mg/L) = $\Delta A / 0.68 \times 1000$, where: (Extinction coefficient) $\epsilon = 0.68$. Total serum protein (TP) concentration was determined by Lowry *et al.*,^[11] method, using BSA as a standard protein. The levels of thiol groups were assayed according to the method of Ellman^[12] which modified by Riddles *et al.*^[15] using the equation below: $A = \epsilon \cdot C \cdot l$, where: $\epsilon = 14,100 \text{ M}^{-1} \text{ CM}^{-1}$. The spectrophotometric estimation of free amino groups was performed according to the Zaia *et al.*,^[16] method, finally, the carbonyl group of the protein was estimated using the method of Levine *et al.*^[17] as in the equation below: $A = \epsilon \cdot C \cdot l$, where: $\epsilon_{370} = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$, and the random blood

sugar (RBS) estimated by the kit supplied from BioLabo company.^[18]

Statistical analysis

Statistical analysis was done using GraphPad Prism Version 6 (GraphPad Software, San Diego, CA, USA). Values were expressed as (mean ± standard deviation [SD]). The comparison of (mean ± SD) was performed using the Student’s *t*-test. Statistical significance was defined as ($P \leq 0.05$) and the correlation between the parameters.

RESULTS

Table 1 summarized the isolation steps of CP in each studied groups; after each isolation step, the “protein content, the CP activity, and specific activity” were estimated.

The results showed that the CP was isolated from serum by using 60% ammonium sulfate (AS) with 54% and (1.2) fold of isolation for both groups, while after dialysis the yield equal to (42 and 39% for patients and healthy controls respectively and (1.4) fold of isolation for each studied groups. Figure 1a and b showed the electrophoresis results of Coomassie stain and Figure 2a and b represents the glycoprotein stain for DM-II patients and healthy controls, respectively.

The electrophoresis results for both “protein and glycoprotein” stain indicated that the bands of CP in patients group have

more intensity than the bands of healthy controls. Table 2 represents the activity of CP and the levels of some biochemical parameters as mean ± SD for all studied groups.

The results showed that there was non-significant ($P \geq 0.05$) increase in the total protein, thiol/protein, carbonyl and carbonyl/protein levels.

The results also showed a non-significant ($P \geq 0.05$) decrease in the free amino and free amino/protein levels, and significant ($P \leq 0.05$) increase in the RBS and thiol levels in patients group compared to healthy controls.

Various phenotype correlations were established between the studied biochemical parameters in patients group. The results indicated that there was a significant ($P \leq 0.05$) positive correlation between CP and RBS with $r = 0.306$ and non-significant ($P \geq 0.05$) correlation coefficients was appeared between the other studied parameters [Table 3].

DISCUSSION

Under normal physiological conditions, there is a balance in the generation of oxygen-free radicals and the antioxidant defense mechanisms used to deactivate free radical toxicity. Impairment in the oxidant/antioxidant equilibrium results in oxidative stress in numerous pathological conditions including

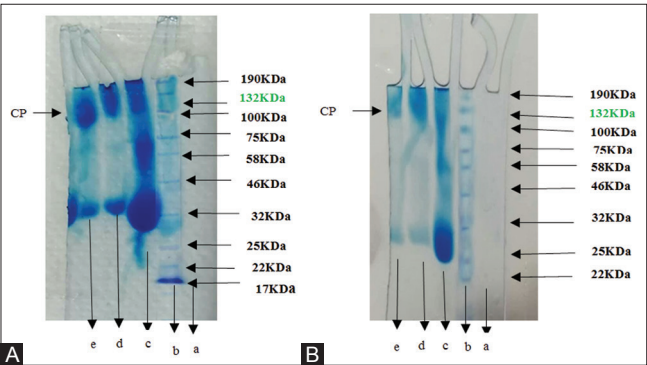


Figure 1: Electrophoretic migration profile of native polyacrylamide gel electrophoresis (10%) with Coomassie stain for diabetes mellitus type II patients. (A) a: Bromo phenol, b: Standard, c: Patient serum, d: Partially purified by AS, e: Partially purified by dialysis. (B) a: Bromo phenol, b: Standard, c: Control serum, d: Partially purified by AS, e: Partially purified by dialysis

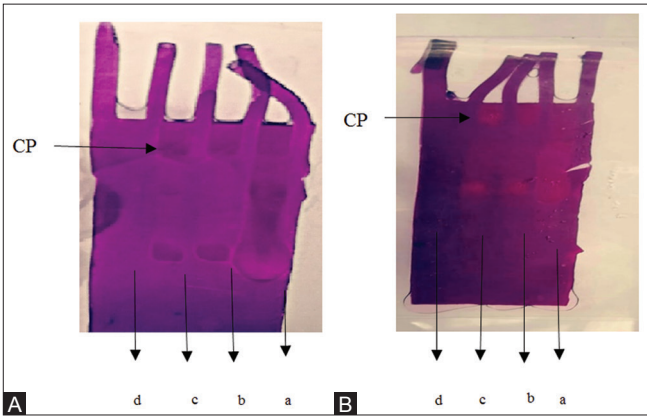


Figure 2: Electrophoretic migration profile of native Polyacrylamide gel electrophoresis (10%) (gel was stained for glycoprotein). (A) a: Patient serum, b: Partially purified by AS, c: Partially purified by dialysis, d: Bromo phenol. (B) a: Control serum, b: Partially purified by AS, c: Partially purified by dialysis, d: Bromo phenol

Table 1: Isolation steps of ceruloplasmin from human serum (diabetes mellitus type II patients and healthy controls)								
Steps	Total volume (ml)	CP (U/ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold of isolation
Patients serum (crude)	3	16.2	32.3	96.9	48.6	0.501	100	1
Ammonium sulfate	1.7	15.3	25.6	43.5	26	0.6	54	1.2
Dialysis	1.4	14.6	20.7	29	20.4	0.703	42	1.4
Healthy serum (crude)	3	18	40	120	54	0.45	100	1
Ammonium sulfate	1.8	16.2	30	54	29	0.54	54	1.2
Dialysis	1.4	15	24	33.6	21	0.63	39	1.4

CP: Ceruloplasmin

Table 2: Activity of ceruloplasmin and the levels of some biochemical parameters as (mean±standard deviation) for all studied groups

Parameters	Mean±SD		P
	Healthy controls (n=25)	DM-II (n=50)	
RBS	88.64±10.01	223.2±92.42	0.0001*
TP (g/dl)	5.632±1.280	5.644±1.645	0.6120
CP (mg/dl)	22.90±7.041	33.05±11.37	0.0129*
Specific activity of CP (U/mg)	0.423±0.143	0.587±0.339	0.0240*
Thiol (μmol/l)	21.84±5.612	22.53±11.06	0.0006*
Thiol (μmol/protein)	4.140±1.601	4.368±2.517	0.6812
Free amino (mmol/l)	0.9791±0.2808	0.8570±0.3000	0.0941
Free amino (mmol/protein)	0.1894±0.07838	0.1653±0.07710	0.2092
Carbonyl (nmol/mg)	77.17±40.36	83.40±42.85	0.5527
Carbonyl (nmol/protein)	14.87±7.145	15.24±8.591	0.8542

*Significant $P \leq 0.05$. CP: Ceruloplasmin, SD: Standard deviation, RBS: Random blood sugar, TP: Total serum protein, DM-II: Diabetes mellitus type II

Table 3: Correlation coefficient between the studied parameters in patients groups

Parameters	DM-II patients r/p
Free amino/carbonyl	0.06340/0.6618
Free amino/thiol	-0.1240/0.3911
Free amino/total protein	-0.04319/0.7659
Carbonyl/glucose	0.1734/0.2285
Carbonyl/thiol	-0.02257/0.8764
Carbonyl/total protein	0.1654/0.2510
CP/carbonyl	0.04369/0.7632
CP/free amino	-0.1047/0.4692
CP/glucose	0.3058/0.0308*
CP/total protein	-0.07345/0.6122
Free amino/glucose	0.1081/0.4549
Thiol/CP	-0.1175/0.4165
Thiol/glucose	0.1665/0.2477
Thiol/total protein	-0.05556/0.7016
Total protein/glucose	0.1039/0.4727

*Significant $P \leq 0.05$. CP: Ceruloplasmin, DM-II: Diabetes mellitus type II

DM leading to cellular damage. Increasing evidence in both experimental and clinical studies suggests that there is a close link between hyperglycemia, oxidative stress, and diabetic complications.^[19]

CP considered as a pro-oxidant or an antioxidant depending on other factors, such as the presence of free ferric ions and ferritin binding sites, acting as a ferroxidase. It is vitally important in regulating the ionic state of iron. Moreover, it probably transports coppers to tissues, which have separate membrane receptors for CP.^[10] In this study the CP activity was increased in patients group compared to healthy controls, these results agreed with Sharma *et al.*^[11] who found that CP was higher in diabetic than nondiabetic group.

Total protein is considered as the most abundant compounds in serum. The proteins are involved enzymes, hormones, and antibodies.^[20] The results indicated that there were increase in the TP levels of the DM-II patients compared to healthy controls these results were consistent with Pasaoglu *et al.*^[21]

who revealed that there was an increase in TP levels in DM-II patients, the increase may be attributed to that blood serum circulates through the tissues, it collects proteins that emitted from their original locations to bound physiological events, including “tissue transforming, trauma, and necrobiosis,” that ends up in a rise within the TP.^[22] Thiol and carbonyl are considered markers of oxidative stress.^[9,23] In this study thiol and carbonyl levels were increased.

Darmaun *et al.*^[24] found that glutathione level that shows a major part of the intracellular thiol level was determined lower in DM-I groups than their age matched controls.^[24] It is thought that low thiol levels may be due to reduction in synthesis or based on usage. However, the increase of thiol in this study agreed with Ihsan^[25] who found that disulfide/native thiol levels were higher than the control group in DM-type I patients. These results showed that in the shift of thiol/disulfide homeostasis toward disulfide form might have a role in both hyperglycemia and inflammation.

The relation between hyperglycemia and aerophilic stress is related to ROS, which are released from glycated proteins caused by hyperglycemia.^[25] The increase in the carbonyl levels agreed with Mateen *et al.*^[23] They found that there was higher protein carbonyl levels in rheumatoid arthritis disease. The increase in the levels of carbonyl is also because of the aerophilous stress and inflammation in patients. Protein carbonyls are formed either by oxidation of certain amino acid residues or by reaction with lipid peroxidation products. The amount of protein carbonyl teams in plasma proteins reveals the intensity of free radical driven reaction and likewise because the extent of protein oxidization.^[23]

The free amino levels in this study was decreased which agreed with Trifunovic-Macedoljan *et al.*^[26] who found that there were decrease in free amine group level in DM disease as compared to healthy controls. Amino, carbonyl, and thiol groups present in the surfaces of proteins participate in protein modification with glucose.^[27,28] The products created in the first phase of the reaction change in a series of subsequent reactions.^[29] Thiol group is a strong nucleophile, at physiological pH values is

stronger than that “amine and carbonyl” groups with Lys and Arg side chains.^[30,31] The potential importance of SH and D-glucose reaction in protein cross-linking has only been reported by Zeng and Davies^[30] and Zeng and Davies,^[32] they suggested that the product of the initial reaction between thiol group and D-glucose can be a target for the next reaction with an amine group or, vice versa, the initial product of a reaction between an amine group and D-glucose can be a target for this group.^[29]

CONCLUSION

The results of this study indicated that the oxidative proteins markers has a role in DM-II compared to healthy controls, which might play an active role in the progression of disease. The findings above may support an association between these oxidation proteins and DM-II. The stronger response observed in serum CP and thiol from patients with the change in the concentration of proteins which suggest that these oxidative protein marker contents may be useful in evaluating the progression of DM-II and in elucidating the mechanisms of disease pathogenesis. The correlation results between CP and RBS which indicated that there were positive correlation between them may be important for evaluation and diagnosis in DM-II patients.

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Conflicts of interest

There are no conflicts of interest.

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