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Original Article

Biochemical Evaluation of Carbonic Anhydrase and Some Antioxidant Markers in Patients with Diabetes Complications

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Abstract

Diabetes is a category of metabolic illnesses defined by a persistently high blood sugar level. This complication is caused by either the pancreas failing to create enough insulin or the body's cells failing to respond correctly to the insulin produced. Diabetes, if left untreated, can lead to a slew of health issues. Diabetic ketoacidosis, hyperosmolar hyperglycemia, and mortality are all examples of acute complications. There are numerous serious long-term consequences, including chronic renal disease, foot ulcers, as well as nerve and eye damage. This study aimed to extract carbonic anhydrase (CA) from human red blood cells and estimate the activity and specific activity of the enzyme and some biochemical parameters, including total protein, albumin, globulin, free amino acids, free amino acids/total protein (TP), thiol, thiol/TP, as well as carbonyl and carbonyl/TP levels in patients with diabetes complications, compared to the healthy subjects; moreover, it was attempted to investigate the correlation among the aforementioned variables. This study included 60 blood samples obtained from patients with diabetes complications and 40 healthy individuals as control. The results revealed a significant ($P \leq 0.05$) decrease in the TP levels, while the CA activity and specific activity were significantly ($P \leq 0.05$) increased. Moreover, there was a non-significant ($P \geq 0.05$) increase in the free amino levels; however, a significant ($P \leq 0.05$) increase was observed in albumin, free amino/TP, thiol, thiol/TP, as well as carbonyl and carbonyl/TP levels. On the other hand, a significant ($P \leq 0.05$) decrease was found in the levels of globulin and albumin/globulin ratio (AGR) in the patients, compared to the healthy subjects. The results also indicated a significant ($P \leq 0.05$) difference in all cases of diabetes mellitus (DM) complications for the measured parameters, except for the TP in DM nephropathy, albumin in cardiovascular disease, free amino in neuropathy and cardiovascular disease, and free amino/TP in retinopathy that showed a clear non-significant ($P \geq 0.05$) difference in the patients' groups, compared to the healthy subjects. The results of correlation indicated a significant ($P \leq 0.05$) positive correlation among free amino/TP, free amino/carbonyl, globulin/TP, and AGR/albumin. However, a significant negative correlation was noted between globulin/albumin and AGR/globulin. The results revealed that the protein oxidation markers and CA as antioxidant markers may play a role in monitoring diabetes complications.

Keywords: Anti-oxidation markers, Carbonic anhydrase, Diabetes complications

1. Introduction

Diabetes mellitus (DM) is one of the fastest-growing diseases in the world, with 693 million people estimated to have it by 2045. Devastating macrovascular effects, such as heart failure, can occur in patients with diabetes (coronary artery disease, peripheral arterial disease, and stroke) (1). Diabetic kidney disease, diabetic retinopathy, and neuropathy are all microvascular disorders that contribute to greater mortality, blindness, kidney failure, and a lower overall

quality of life. (2). The development of diabetic complications, both microvascular and cardiovascular, is influenced by oxidative stress (3). The incidence of DM has been linked to oxidative stress. Several studies have found that oxidative stress plays an important role in the onset and progression of diabetes and related consequences (4). Brownlee (5) shares this viewpoint as "Previously, I proposed oxidative stress as a major role in the development and effects of diabetes". Oxidative stress occurs when the cell's redox balance is disrupted, causing damage to membranes and essential macromolecules including DNA, proteins, and lipids. The two key mechanisms malfunctioning during DM, insulin secretion and insulin action, have been demonstrated to be harmed by oxidative stress. The role of oxidative stress in DM can be compared to the two sides of a coin. The procedure not only accelerates the onset of diabetes but also exacerbates the disease's symptoms and effects (4). Carbonic anhydrase (CA) is a zinc metalloenzyme that predominantly catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+ in living organisms as in the equation $H^+ + HCO_3^- \rightarrow CO_2 + H_2O$. It has a role in a variety of physiological and pathological processes, including electrolyte secretion and biosynthetic reactions, such as gluconeogenesis, lipogenesis, and ureagenesis. CA is the major driver of hepatic gluconeogenesis (6), and carbonic anhydrase inhibitors (CAI) is a cytosolic enzyme with low carbon dioxide hydratase activity that may scavenge oxygen free radicals *in vivo* and protect cells from oxidative damage. The presence of CA speeds the dehydration of HCO_3^-, thereby ensuring rapid equilibrium between CO_2 species since this is such an important reaction in life. Since Meldrum and Roughton discovered CA in red blood cells (RBCs) about 90 years ago, researchers have been studying its physiological role (7). CA was discovered during a time when physiologists were particularly interested in the chemical makeup of blood. At least one CA family (α, β, γ, δ, Ɛ, ŋ, and ɵ) and each Virtua organism contain at least one CA family (8). Moreover, some early evidence suggested changes in the CA activity in human RBCs, and it is possible that this is the first sign of a changing metabolism in DM (9, 10); however, the precise role of CA in the pathogenesis of DM type I or DM type II is completely unknown. Albumin and globulin are two main components of human serum proteins, and the albumin/globulin ratio (AGR) can be calculated by comparing total protein (TP) and albumin levels. They are important for immunity and inflammation all across the body. The AGR can be used to determine the prognosis of cancer patients (9). The AGR has become one of the most important parameters that play an important role in diagnosing diseases, as in a study, they found that AGR could predict the mortality risk in patients with chronic kidney disease (10). In addition, the diabetic rats had decreased levels of plasma total protein, albumin, globulin, and AGR, compared to the control rats (11). Amino acids play critical roles in a variety of metabolic processes, and measuring free amino acids in biological fluids and tissues has long been used to offer nutritional information for the diagnosis of disorders, particularly metabolic deficits. Plasma free amino acids (PFAAs), particularly branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs), have been associated with visceral obesity, insulin resistance, and MD in several investigations. After controlling body mass index, insulin resistance was linked to changes in PFAA levels (e.g., BCAAs and AAAs), a cluster of BCAAs, and related amino acids. Despite the fact that circulating fatty acids and inflammatory cytokines are well-known risk factors for insulin resistance, they had no effect on insulin dysregulation (12).

Through oxidation, reduction, and disulfide exchange, thiol groups play an important role in signaling and homeostasis. Single tubs of tiny molecules (e.g., cysteine), peptides (e.g., glutathione), and thiol proteins (e.g., thioredoxin) that are not in balance and have particular oxidized/reduced ratios, making up the total thiol pool (13). Thiols are molecules with the sulfhydryl group-SH attached to one of their carbon atoms. They are endogenous chemicals that assist aerobic cells to retain a reduced state despite an oxidizing environment

(14). Due to their ability to react with free radicals, thiols are very efficient antioxidants that protect cells from free radical damage (15). Carbonyl groups are formed when proteins are oxidized, and their presence in tissues and plasma is a rather persistent indicator of oxidative damage (16). The enhanced chemical alteration of proteins by carbohydrates and lipids in DM is due to the routes of metabolism participating in the reactive carbonyl species detoxification being overloaded, as a result, both oxidative and nonoxidative reactions produce more reactive carbonyl molecules at the steady-state levels (17). Protein carbonyl concentration is the most extensively used and most generic biomarker of protein oxidation (18). Hyperglycemia undoubtedly causes increased oxidative stress, with carbonyl as one of its marks, if any, in the diabetic group (19). Protein carbonylation is a kind of protein oxidation that can be enhanced by reactive oxygen species (ROS), which can interact directly with proteins or produce products (reactive carbonyl species) and then interact with proteins (20). The oxidation of the side chains of lysine, arginine, proline, and threonine residues or the oxidation of the side chains of arginine, proline, and threonine residues provides extremely reactive carbonyl derivatives when proteins are directly oxidized by ROS or from the oxidation of the side chains of arginine, proline, and threonine residues or the oxidation of the side chains of arginine, proline, and threonine residues (20). This study aimed to estimate the activity and specific activity of human erythrocytes CA, TP, albumin, globulin, AGR, free amino, free amino/TP, thiol, thiol/TP, as well as carbonyl and carbonyl/TP levels in patients with diabetes complications, compared to the healthy subjects; moreover, it was attempted to investigate the correlation coefficient among them in the patients' group.

2. Materials and Methods

A total of 100 blood samples were investigated in this study. The DM patient group included 60 blood samples (36 males and 24 females) with complications, such as retinopathy, nephropathy, neuropathy, and cardiovascular disease, with an age range from 30 to 70 years. These patients visited a specialist center, Kirkuk, Iraq, from October 2020 to January 2021 and were diagnosed by specialists. Other 40 blood samples of healthy subjects were also included in this study and regarded as the control group, with the same age range as the patient group.

Totally, 3 mL of blood was collected by venipuncture in an EDTA tube for separation using a disposable syringe. The tubes were centrifuged at 1500×g for 15 min. After separating the plasma from the RBC samples, the RBCs were washed with (1 mL) normal saline and centrifuged at 1500×g for 10 min. This process was repeated three times, and then deionized water was added to twice the volume of blood. RBC samples were stored at -20˚C to be used on the second day (21).

2.1. Biochemical Assays

As mentioned by Ibrahim , Amodu (22), (23), CA activity was assessed, with the change proposed by Parui, Gambhir (24). The esterase activity of CA was measured using the rate of hydrolysis of (3mM) p-nitrophenyl acetate to p-nitrophenol in this test (24) using p-nitrophenyl acetate as substrate. The activity of CA is calculated according to the following equation: CA activity=$(\Delta A/3min\times50070)\times125\times10^{6}\times10^{-3}$(µmole/min/mL) where: (Extinction coefficient) ε=50070 $cm^{-1}M^{-1}$. The concentration of total erythrocyte protein was measured using the technique by Lowry, Rosebrough (25) and BSA as a standard protein. The spectrophotometric determination of free amino groups was performed according to the method by Zaia, Barreto (26) The concentrations of thiol groups were estimated according to the Ellman method (27), which was modified by Riddles, Blakeley (28) using the equation: A=ε.C.l, where: ε=14,100 $M^{-1}cm^{-1}$. The protein carbonyl content was assayed according to the method of Levine, Garland (29) as in the equation: A=ε .C. l, where: ε_{370} =22,000 $M^{-1}cm^{-1}$.

Finally, albumin concentration was estimated by method (30) using the equation: Alb conc. (g/dl)=(Abs sample/Abs standard)×standard conc. (4g/dl).

2.2. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism software (version 8, San Diego, CA, USA). Values were expressed as mean±SD. The comparison of mean±SD was performed using the ANOVA. A *P*-value of ≤0.05 was considered statistically significant.

3. Results

Tables 1 and 2 represent the TP levels, activity, and specific activity of CA as mean±SD for the two studied groups. Moreover, the patient group was divided into retinopathy, nephropathy, neuropathy, and cardiovascular disease, compared to the healthy subjects.

The results in table 1 indicate a significant ($P \leq 0.05$) decrease in the TP levels; however, the CA activity and specific activity were significantly ($P \leq 0.05$) increased in the patients' group, compared to the healthy subjects. As can be observed in table 2, the TP levels were significantly ($P \leq 0.05$) decreased in all patients' groups, except for those with kidney disease, which was non-significantly ($P \geq 0.05$) decreased; furthermore, the activity and specific activity of the CA results indicated a significant ($P \leq 0.05$) increase in all patients' groups, compared to the healthy subjects. Table 3 tabulates the levels of some biochemical parameters as mean±SD for the two studied groups.

Table 1. TP, as well as activity and specific activity of CA as mean±SD for the two studied groups

Parameters	Healthy subjects (n=40) (mean±SD)	Patients (n=60) (mean±SD)	*P*- value
TP (g/dl)	10.16±1.44	8.302±1.57	$P \leq 0.05$
CA Activity (U/ml)	1.44±0.29	3.406±0.49	$P \leq 0.05$
CA specific activity (U/mg)	0.015±0.003	0.044±0.009	$P \leq 0.05$

Table 2. TP, as well as activity and specific activity of CA as mean± SD for all studied groups

Parameters	Healthy controls (n=40) (mean±SD)	Retinopathy (n=15) (mean±SD)	Kidney disease (n=15) (mean±SD)	Neuropathy (n=15) (mean±SD)	Cardiovascular disease (n=15) (mean±SD)
TP (g/dl)	10.15±1.44[a]	7.87±1.30[b]	9.357±0.76[a]	8.800±1.38[c]	7.17±1.77[d]
CA activity (U/ml)	1.44±0.29[a]	3.48±0.60[b]	3.370±0.46[c]	3.223±0.58[d]	3.56±0.22[e]
CA specific activity (U/mg)	0.015±0.003[a]	0.044±0.008[b]	0.043±0.005[c]	0.04±0.008[d]	0.05±0.013[e]

The different letters refer to significant differences

Table 3. Levels of some biochemical parameters as mean±SD for the two studied groups

Parameters	Healthy subjects (n=40) (mean±SD)	Patients (n=60) (mean±SD)	*P* -value
Albumin(mg/dl)	3.93±0.46	5.19±1.65	$P \leq 0.05$
Globulin(mg/dl)	6.27±1.43	3.09±1.93	$P \leq 0.05$
Albumin/globulin ratio	0.65±0.16	4.39±9.63	$P \leq 0.05$
Free amino (mM)	455.5±56.24	459.8±84.13	$P \geq 0.05$
Free amino/TP 10^3(mmol/mg)	4.58±0.90	5.76±1.25	$P \leq 0.05$
Thiol (µM)	2580±531.3	3044±109.2	$P \leq 0.05$
Thiol/TP10^3 (µmol/mg)	26.20±6.98	38.83±9.35	$P \leq 0.05$
Carbonyl (nM)	1188±309.5	1524±481.3	$P \leq 0.05$
Carbonyl/ TP10^3(nM/mg)	11.81±03.27	19.19±7.17	$P \leq 0.05$

The results in table 3 indicate a non-significant ($P \geq 0.05$) increase in the free amino levels; however, it shows a significant ($P \leq 0.05$) increase in albumin, free amino/TP, thiol, thiol/TP, as well as carbonyl and carbonyl/TP levels. A significant ($P \leq 0.05$) decrease was also observed in the levels of globulin and AGR in the patients' groups, compared to the healthy subjects. Table 4 summarizes the levels of albumin, globulin, AGR, free amino, free amino/TP, thiol, thiol/TP, as well as carbonyl and carbonyl/TP as mean±SD for patients with DM complications subdivided into groups of retinopathy, nephropathy, neuropathy, and cardiovascular disease, compared to the healthy subjects.

The results indicate a significant ($P \leq 0.05$) difference in all cases of DM complications for the following measured parameters, except for the TP in DM nephropathy, albumin in cardiovascular disease, free amino in neuropathy and cardiovascular disease, and free amino/TP in retinopathy that they showed s non-significant ($P \geq 0.05$) difference. This study also estimated the correlation coefficient among the studied parameters in the patients' groups (Table 5).

Table 4. Albumin, globulin, albumin/globulin ratio, free amino, free amino/TP, thiol, thiol/TP, as well as carbonyl and carbonyl/TP for five groups of patients, compared to the healthy subjects

Parameter	Healthy controls (n=40) (mean ±SD)	Retinopathy (n=15) (mean±SD)	Kidney damage (n=15) (mean±SD)	Neuropathy (n=15) (mean±SD)	Cardiovascular disease (n=15) (mean±SD)
Albumin (mg/dl)	3.93±0.46^{a}	5.27±1.70^{b}	6.25±1.41^{c}	4.88±1.49^{d}	4.31±1.50^{a}
Globulin(mg/dl)	6.27±1.43^{a}	2.46±1.39^{b}	3.40±1.35^{c}	3.42±1.71^{d}	3.11±2.90^{e}
Albumin/globulin ratio	0.65±0.16^{a}	5.99±12.9^{a}	2.88±3.17^{a}	1.79±0.87^{a}	6.92±13.9^{b}
Free amino (mM)	455.5±56.2^{a}	390.2±73.7^{b}	521.1±56.8^{c}	491.3±81.8^{a}	436.5±60.0^{a}
Free amino/TP (mmole/mg)	4.58±0.90^{a}	4.97±0.68^{a}	5.60±0.84^{b}	6.12±1.64^{c}	6.33±1.22^{d}
Thiol (µM)	2580±531.3^{a}	3004±84.86^{b}	3063±120.6^{c}	3036±120.8^{d}	3073±104.4^{e}
Thiol/TP(µmole/mg)	26.20±6.98^{a}	39.23±6.93^{b}	32.64±3.42^{c}	38.23±10.6^{d}	45.22±10.5^{e}
Carbonyl (nM)	1188±309.5^{a}	1345±377.3^{b}	1474±451.4^{c}	1476±317.0^{d}	1800±639.2^{e}
Carbonyl/TP(nmole/mg)	11.81±3.26^{a}	17.85±6.89^{b}	15.80±4.78^{c}	17.37±5.54^{d}	25.74±7.17^{e}

The different letters refer to significant differences

Table 5. Correlation coefficient among the studied parameters in the patients' groups

Parameters	(r/p)	Parameters	(r/p)
CA/TP	0.19/0.15	TP/AGR	-0.22/0.0965
CA/free amino	0.077/0.56	Free amino/albumin	0.15/0.27
CA/thiol	-0.082/0.53	Free amino/globulin	0.16/0.23
CA/carbonyl	0.11/0.40	Free amino/AGR	-0.04/0.75
free amino/ TP	0.42/0.001	Thiol/albumin	0.05/0.73
Thiol/ TP	-0.049/0.71	Thiol/globulin	0.14/0.29
Carbonyl/ TP	-0.027/0.84	Thiol/AGR	-0.03/0.82
Free amino/thiol	0.070/0.59	Carbonyl/albumin	-0.25/0.05
Free amino/carbonyl	0.28/0.03	Carbonyl/globulin	0.21/0.10
Globulin/albumin	-0.43/0.001	Carbonyl/AGR	0.005/0.97
Albumin/ TP	0.18/0.18	AGR/albumin	0.32/0.012
Globulin/ TP	0.57/0.0001	AGR/globulin	-0.46/0.0002

$P \leq 0.05$ significant; $P \geq 0.05$ non-significant; r=Correlation Coefficient
CA: Carbonic anhydrase; AGR: Albumin/globulin ratio; TP: Total protein

The results of the correlation indicated a significant ($P \leq 0.05$) positive association among free amino/TP, free amino/carbonyl, globulin/TP, and AGR/ albumin; however, a significant negative correlation was observed between globulin/albumin and AGR/globulin. There was a non-significant ($P \leq 0.05$) correlation among globulin/albumin, carbonyl/albumin, and AGR/ globulin.

4. Discussion

In DM, drastic changes occur in the human body, and significant changes in the patient's biochemistry are part of that process which reflects the body's physiology. Although various etiologies are responsible for the heterogeneous disease group of DM, all patients share abnormalities in carbohydrate, fat, and protein metabolism. Long-term DM affects many organ systems (31). The results of activity and specific activity of CA in this study may be due to a rise in blood glucose concentration in diabetic patients, which induced a higher glycolytic rate in RBCs, and as a result, a higher lactate concentration, which induced CA activity (6, 22). Ibrahim, Amodu (22) compared the activity of CA isolated from the RBCs of DM type II patients with healthy participants and found a substantial significant increase in CA activity. By dividing the activity of CA on the concentration of TP, the significant increase in specific activity can be attributed to the removal of the impact of some impurities present in the RBCs extract, making specific activity a measure of enzyme purity (32). Hussein and Zainal (33) studied CA in the RBCs of patients with β-Thalassemia and found a decrease in the activity of CA. The results of TP indicated a significant decrease in the patients' groups, compared to the healthy subjects and in the patients subdivided into groups, except for those with nephropathy in whom the TP was non-significantly decreased. The TP is one of the most prevalent molecules present as enzymes, hormones, and antibodies; moreover, it acts as osmotic pressure regulators. A reduction in the liver's ability to synthesize protein might be a secondary cause of lower TP levels. Albumin is the most abundant protein in the blood (usually over 50%). It is produced by the liver and aids in the control of osmotic pressure, nutrition transport, and waste disposal (33). The lack of natural feedback inhibition of gluconeogenesis in the liver causes an increase in the breakdown of lipids and proteins, as well as the conversion of glucogenic amino acids to glucose, resulting in an increase in glucose levels that may be due to hemodilution which can be regarded as the cause of the decline. The results of this study agreed with the findings of a study conducted by Al-Muhtaseb (34). They found a decrease in the TP levels when they looked at serum and/or tissue samples from patients with breast cancer and gynecological malignancies. Albumin has been demonstrated to have a crucial function in the blood plasma's anti-oxidative ability against ROS (33). In this study albumin significantly increased in patients, compared to the healthy subjects. The results from the albumin levels were not in accordance with the findings of a study by Yassin, Soliman (35). Globulins are a secondary component of complete serum proteins that serve as carriers for sex hormones and play an important function in immunity and inflammation Toiyama, Yasuda (36).According to the results of globulin levels, they were significantly decreased in patients, compared to the healthy subjects. The possible reason for the decrease in the globulin was the excessive sugar and the natural filtration of the kidneys changes, which leads to the collection of toxic wastes, a large amount of which is lost with urine (32). Albumin and globulin levels, as well as the AGR, are easily detectable biomarkers that may be used in conjunction to predict the patients' survival in a variety of illnesses. The AGR representative parameters are used to measure systemic inflammation rank (37), and the results indicated a significant increase in the patients, compared to healthy controls. The results also indicated a non-significant increase in free amino and free amino/TP levels in patients, compared to the healthy subjects. These results disagree with the findings of a study by Trifunović-Macedoljan, Pantelić (38). They found a

decrease in free amine group levels in DM patients, compared to the healthy subjects and the results of a study by Saleem, Dahpy (39).The reason for increased levels of amino groups may be due to the elevated circulating levels of BCAAs and the central nervous system (Acyl CNsO) that are associated with fatty acids in DM patients Mihalik, Michaliszyn (40).

The results of thiol and thiol/TP levels in all patients' groups indicated a significant increase, compared to the healthy subjects. These results are in line with the findings of a study by Ates, Kaplan (41); however, they are not consistent with the results of the studies performed bySener, Akbas (42), Ergin Tuncay, Erkilic (43) and Darmaun, Smith (44). They investigated the rosacea disease, and the two later studies assessed DM type 1, respectively. They found that disulfide/native thiol levels were greater in patients with DM type 1 disulfide, compared to the control group. These findings suggested that hyperglycemia and inflammation may both have a role. Hyperglycemia and aerophilic stress are linked by ROS which is generated from glycated proteins as a result of hyperglycemia (41, 45). As for the carbonyl and carbonyl/TP levels and all patients' groups, the results indicated a significant increase in patients with diabetes complications, compared to the healthy subjects. These results agreed with the findings of a study by Abd and Zainal (46), who found an increase in carbonyl/TP levels in β-thalassemia patients. The findings are also in line with the results of a study byMateen, Moin (47) and Odetti, Garibaldi (48) the increase in carbonyl level may be attributed to the patients' aerophilic stress and inflammation (49). In DM type II patients, protein oxidation indicators, such as protein carbonyl and thiol group levels have been discovered. Protein carbonyls are formed when certain amino acid residues are oxidized or when lipid peroxidation products interact with proteins. The structure and function of proteins, enzymes, and membranes are maintained by protein carbonyl and thiol group levels reported in DM type II patients due to ROS-mediated oxidation of proteins.

They can minimize the damage caused by oxidative stress if they work together (47, 49). At oxidation, carbonyl groups (aldehydes and ketones) are formed on the side chains of proteins, particularly Pro, Arg, Lys, and Thr. Chemical stability is advantageous in order to detect and store these moieties. Carbonyl derivatives of proteins can also be generated by oxidative cleavage of proteins via the -amidation pathway or by the oxidation of glutamyl side chains, resulting in a peptide with a -ketoacyl derivative blocking the N-terminal amino acid. Protein carbonyl concentration is actually the most common and widely utilized biomarker of protein oxidation (50). The findings of this study revealed that oxidative protein indicators may play a role in diabetic complications, compared to the healthy subjects, suggesting that they may play a function in disease development. Correlation studies among the investigated parameters indicated that the results of this study of oxidative markers may have an active role in diabetic complications, which could play a role in disease progression. The evidence presented above could also point to a link between these oxidation markers and diabetic complications which may be important for the evaluation and diagnosis of patients with diabetic complications.

Authors' Contribution

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

Ethics

All studies were performed in compliance with the rules of the human ethics of the University of Kirkuk, Kirkuk, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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