Association of C-peptide with immunological and biochemical parameters in patients with type 1 diabetes mellitus

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Abstract. Type 1 diabetes mellitus (T1DM) is an autoimmune metabolic disorder characterized by the destruction of pancreatic β-cells due to the autoimmune reactions of autoreactive T-cells and autoantibodies. The present study aimed to investigate the association between the levels of connecting peptide (C-peptide), and several biochemical and immunological markers in patients with T1DM treated with insulin. For this purpose, a total of 5 ml venous blood was collected from 44 patients with T1DM and 28 healthy volunteers. Relevant clinical examinations were carried out. The contents of C-peptide, IL-4 and IL-5 were determined using the corresponding ELISA kits. The normal ranges for liver and renal function tests were set for both patients and controls. The results revealed that the mean concentrations of hydrogen peroxide (H_2O_2) was higher in patients with T1DM compared with healthy controls. However, the difference was not statistically significant. In addition, the levels of IL-5 were decreased in patients with T1DM compared with healthy individuals. No statistically significant correlation was observed between the levels of C-peptide and cytokines with the exception of IL-4 in healthy volunteers, which were positively correlated with those of C-peptide. Overall, the results demonstrated that the low C-peptide levels in patients with T1DM were not associated with the measured parameters. However, patients with T1DM could still be at high risk of developing pathological complications due to the enhanced levels of glucose and H_2O_2, and the decreased levels of IL-5.

Introduction

Type 1 diabetes mellitus (T1DM) or insulin-dependent diabetes mellitus is an autoimmune disease characterized by the interaction between the innate and adaptive immune system with pancreatic β-cells, thus resulting in a complex pathogenesis. The incidence and prevalence of T1DM are increasing globally, with an annual increase of 2-5% (1-3). The highest incidence of T1DM is observed among children <15 years of age (4). The increasing incidence of T1DM has been associated with environmental and genetic factors or with gene-environment interactions (5).

T-cells play a crucial role in the expansion of autoimmune β-cells and in the development and manifestation of diabetes. However, the specific mechanisms involved in the activation and migration of T-cells to β-cell islets warrant further investigation. Previous studies have suggested that in addition to chemokines and cytokines produced during immune responses, autoantigens may also be involved in the aforementioned process (6-8).

Autoreactive $CD4^+$ T-cells can recognize peptides expressed on β-cells presented by antigen-presenting cells to activate autoreactive $CD8^+$ T cells to lyse β-cells (5,9). The aforementioned process promotes the destruction and loss of insulin-producing β-cells, which in turn leads to insulin deficiency in patients with T1DM, eventually resulting in impaired glucose metabolism (6). Although the destruction of β-cells is mediated by autoreactive T-cells, the development of islet autoantibodies is considered a risk factor for the onset of T1DM (10,11). Several types of autoantibodies can rise in T1DM, such as insulin autoantibodies, islet β-cell antibodies and glutamic acid decarboxylase autoantibodies, which are commonly used in clinical practice to diagnose T1DM (11).

Although the prevailing conviction is that T-cells are the key players in mediating T1DM, emerging evidence has also suggested the significant effect B-cells on the development of the disease (12). The significant effect of B-cells in autoimmunity has been supported by their function as antigen-presenting cells mediated by their interaction with $CD4^+$ or $CD8^+$ cells and the secretion of inflammatory cytokines (12,13).

The proinsulin (PI) polypeptide, which is composed of the A and B chains, the insulin core and the connecting peptide (C-peptide), transfers to the Golgi apparatus and is then cleaved into insulin and C-peptide (14). Therefore, the C-peptide is a by-product produced by the enzymatic conversion of proinsulin to insulin (15). It has been reported that

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the C-peptide has several functions, such as anti-apoptotic, anti-inflammatory and cytoprotective effects. Of note, a previous study demonstrated that C-peptide inhibited the production of reactive oxygen species (ROS), thus attenuating cell apoptosis (16).

It has also been reported that the levels of C-peptide are diminished in the majority of patients with T1DM, thus negatively affecting these patients. Despite medical care, these negative effects may be associated with several biochemical and immunological parameters in patients with T1DM. Through a considerable number of experiments, several studies have provided a large amount of data on the biology of C-peptide (14-16). However, further data in clinical practice are urgently required. Therefore, the present study aimed to investigate the association between the levels of C-peptide, and the biochemical and immunological parameters of insulin-treated patients with T1DM.

Materials and methods

Materials. The Cobas-GLUC3 kit (ref. no. 04404483190), Cobas-A1C kit (ref. no. 05336180190), Cobas-ASTL kit (ref. no. 20764949322), Cobas-ALTL kit (ref. no. 20764957322), Cobas-ALPTL kit (ref. no. 03333752190), Cobas-UREAL kit (ref. no. 04460715190) and Cobas-CREG2 (ref. no. 04810716190) were provided by Roche Diagnostics GmbH. The Ca kit (cat. no. MAK022), Fe kit (cat. no. MAK025) and H_2O_2 kit (cat. no. MAK311) were provided by MilliporeSigma. The C-peptide kit (cat. no. E-EL-H6020), IL-4 kit (cat. no. E-EL-H0101), and IL-5 kit (cat. no. E-EL-H0191) were provided by Elabscience Biotechnology, Inc.

Study design. The present study was a cross-sectional study and was performed at the postgraduate laboratories at the Biology Department, College of Science, University of Kerbala in cooperation with the laboratory of Imam Al-Hassan Diabetes and Endocrinology Specialized Center, Imam Hussain Medical City, Iraq.

Study population. In the present study, 72 individuals, including 44 patients already diagnosed with T1DM [25 (57%) females and 19 (43%) males], and 28 healthy volunteers [HVs; 15 (54%) females and 13 (46%) males] were enrolled using the convenience sampling method (from December, 2020 to June, 2021). The age range of the subjects was 7-25 years. All participants or their parents, in the case of minors, provided written informed consent to participate in the study. The data including age, sex, onset of disease, family history, T1DM treatment and complications were recorded using a questionnaire. The patients and HVs, > years of age, pregnant women, patients with genetical systemic and respiratory diseases, and patients with liver failure, renal impairment, tumors and mentally illness were excluded from the study. In addition, based on the data recorded in the questionnaire and a physical examination, individuals with microvascular complications such as diabetic kidney disease, retinopathy and neuropathy, patients with macrovascular complications such as hypertension and dyslipidemia, and those with autoimmune diseases, were also excluded. Therefore, only patients with T1DM with no pathological complications, at >1 year after diagnosis, with diabetes and undergoing insulin treatment were included in the present study.

Methods. The T1DM diagnostic parameters include fasting blood glucose (FBG; cut-off point, 100 mg/dl); glycated hemoglobin (HbA1c; cut-off point, 5.7%) (17); liver function tests, such as aspartate transaminase (AST; cut-off point, 60 U/l), alanine aminotransferase (ALT; cut-off point, 45 U/l) and alkaline phosphatase (ALP; cut-off point, 350 U/l) (18); renal function tests; urea (cut off point, 20 mg/dl); and creatinine (cut-off point, 1.2 mg/dl) (19). These parameters were examined using an automated method, Cobas modular analyzer series (Roche Diagnostics GmbH).

The levels of biochemical parameters, such as serum calcium (S-Ca; cut-off point, 10.9 mg/dl) (20), iron (Fe; cut-off point, 101 μg/dl) (21) and hydrogen peroxide (H_2O_2; cut-off point, 85 mmol/l) (22) were examined using a colorimetric assay kit (MilliporeSigma) and a UV/Vis. Spectrophotometer (Shimadzu Corporation).

The concentrations of C-peptide, IL-4 and IL-5 were estimated in the sera of all participants using ELISA (Elisys Uno device, ref. no. 17350, HUMAN Diagnostics Worldwide).

Sampling evaluation study. A total of 72 (44 T1DM and 28 HV) blood samples were intravenously withdrawn from the patients and controls. The time of blood collection for fasting diabetic patients and controls was from 9 a.m. to 12 p.m. A total of 5 ml venous blood was withdrawn from each participant, and each blood sample was divided into two sections; 2 ml were transformed into ethylenediamine tetra acetic acid (EDTA) tubes, and the remaining 3 ml were pushed gently into a gel tube. The sera were separated following centrifugation of the blood in the gel tubes at 4,000 x g for 10 min at 4˚C. The sera were divided into aliquots and stored at temperatures below -20˚C until use.

Anthropometry. The anthropometric assessment of the body, which involved the body mass index (BMI in kg/m^2; cut-off point, 24.9 kg/m^2), height and weight were documented during the physical examination.

Statistical analysis. All statistical analyses were carried out using GraphPad prism 8 software (GraphPad Software, Inc.). The data were assessed for normal distribution. The differences between two groups, with normally distributed data, were compared using an unpaired Student's t-test for independent samples. To compare the differences between pairs of non-normally distributed data, the Mann-Whitney test was used. Spearman's correlation analysis was performed to evaluate the correlation between two non-normally distributed variables [for T1DM: C-peptide with blood urea nitrogen (BUN), ALP, ALT, AST, serum calcium, IL-4 and IL-5; for HVs: C-peptide with ALT, serum calcium, serum Fe, H_2O_2, IL-4 and IL-5], while the correlation between two normally distributed variables were assessed using Pearson's correlation analysis (for T1DM: C-peptide with FBS, HbA1c, BMI, serum Creatinine, serum Fe and H_2O_2; for HVs: C-peptide with FBS, HbA1c, BMI, BUN, serum creatinine, ALP and AST). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Demographic parameters and comparisons between patients with T1DM and healthy volunteers as regards biochemical parameters and cytokine concentrations.

Parameter	T1DM (n=44)	Healthy volunteers (n=28)	P-value
Sex, n (%)			-
Females	25 (57%)	15 (54%)	
Males	19 (43%)	13 (46%)	
Age, years, n (%)			-
<15	23 (52%)	14 (50%)	
≥15	21 (48%)	14 (50%)	
FBG, mg/dl	311.9±138.5	101.2±9.16	<0.0001
HbA1c, %	10.32±2.07	4.89±0.31	<0.0001
BMI, kg/m^2	20.26±4.12	20.86±4.16	0.57
AST, U/l	21.39±7.62	31.35±9.58	<0.0001
ALT, U/l	16.98±10.54	15.80±6.09	0.78
ALP, U/l	276.7±203.9	517.4±291.0	0.0005
BUN, mg/dl	23.26±7.02	28.96±6.79	0.0002
Serum creatinine, mg/l	0.52±0.16	0.60±0.12	0.018
Serum Ca, mg/dl	9.47±0.59	8.89±0.75	<0.0001
Serum Fe, μg/dl	78.10±34.70	159.2±121.9	0.003
H_2O_2, mmol/l	76.15±26.77	70.28±39.80	0.46
C-peptide, pg/ml	11.33±23.46	84.23±90.59	<0.0001
IL-4, pg/ml	25.04±5.18	27.40±8.45	0.19
IL-5, pg/ml	46.05±44.87	61.30±41.87	0.04

Values are presented as the mean ± SD. P<0.05, was considered to indicate a statistically significant difference. T1DM, type 1 diabetes mellitus; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; BMI, body mass index; AST, aspartate transaminase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; Ca, calcium; Fe, iron; H_2O_2, hydrogen peroxide; C-peptide, connecting peptide.

Results

Blood glucose levels are increased and iron levels were decreased in patients with T1DM. In the present study, 44 patients with T1DM and 28 HVs were enrolled. The differences between the T1DM and HV groups are presented in Table I. Therefore, blood glucose levels, including FBG and HbA1c levels, were significantly higher in patients with T1DM compared with the HVs. Additionally, iron levels were notably decreased in patients with T1DM, while the BMI was equivalent in both groups.

Liver and kidney functions remain unaltered in patients with T1DM. The liver function test values, including ALT, AST and ALP were within the normal range in both groups. The same trend was obtained in the kidney function test, as reflected by BUN and serum creatinine levels. Therefore, BUN and serum creatinine levels were within the normal range in both groups. Despite the fact that the liver and kidney function values were within the normal ranges in both groups, significant differences were found in some of these values, as shown in Table I.

The calcium and H_2O_2 levels were enhanced, and the Fe levels were decreased in patients with T1DM. Calcium and iron concentrations were determined in the sera of patients with T1DM and HVs. The analysis revealed that the calcium concentration was notably elevated, while the Fe levels were significantly decreased in patients with T1DM compared with the HVs (P<0.0001 and P=0.003, respectively). Additionally, the levels of H_2O_2 were higher in the sera of patients with T1DM. However, the difference was not statistically significant (P=0.46).

The C-peptide levels were not associated with the IL-4, IL-5 and H_2O_2 levels in patients with T1DM. The levels of C-peptide were significantly lower in the T1DM group compared with the HV group (P<0.0001). In addition, no statistically significant difference was found in the serum levels of IL-4 between the two groups (P=0.19). However, the IL-5 levels were notably higher in the HVs compared with the patients with T1DM (P=0.04) (Table I).

Furthermore, the results revealed that there were no significant correlations between the C-peptide levels and the biochemical parameters of both patients with T1DM and HVs. Therefore, only mildly inverse or positive correlations were observed. Additionally, the C-peptide levels weakly and negatively correlated with the H_2O_2 levels only in the HVs. However, no statistically significant difference was found (P=0.29; Table II). In addition, as shown in Table III, a moderately significant correlation was obtained between the IL-4 and C-peptide levels only in the HVs (P=0.03). Finally, an inverse correlation was observed between the two immune markers, IL-4 and IL-5, and the C-peptide levels in patients with T1DM. However, no statistically significant differences were observed.

Discussion

The present study aimed to explore the association between C-peptide levels, and different biochemical and immunological parameters in patients with T1DM treated with insulin. Patients with T1DM displayed values within the normal range in the majority of the parameters measured, including (liver and renal tests and calcium concentration). However, other critical parameters, such as FBG, HbA1c and C-peptide levels may be associated with a high risk of T1DM onset and a poor control of the disease in patients treated with insulin. Herein, the Fe levels were significantly decreased in patients with T1DM, which is a common finding in such patients. Although the levels of H_2O_2 were higher in patients with T1DM, no statistically significant difference was observed compared with the HV group. In terms of immunological markers, IL-5 levels were significantly lower in the patients with T1DM compared with the HVs.

The number of studies on patients with T1DM, who have already been diagnosed with diabetes and treated with insulin, is limited. In these studies, no significant differences were obtained between patients with T1DM and the controls in terms of demographic characteristics, such as sex and BMI, as well as biochemical characteristics, including BUN and serum creatinine levels (23-25).

Table II. Correlations between C-peptide and biochemical parameters in patients with T1DM and healthy volunteers.

	C-peptide		
Parameter	Correlation coefficient and P-value	T1DM	Healthy volunteers
FBS	R/Rs value	-0.17 (R)	0.11 (R)
	P-value	0.28	0.58
HbA1c	R/Rs value	-0.20 (R)	0.19 (R)
	P-value	0.19	0.32
BMI	R/Rs value	0.05 (R)	-0.19 (R)
	P-value	0.74	0.33
AST	R/Rs value	0.04 (Rs)	0.09 (R)
	P-value	0.77	0.61
ALT	R/Rs value	0.04 (Rs)	-0.15 (Rs)
	P-value	0.80	0.45
ALP	R/Rs value	-0.14 (Rs)	-0.14 (R)
	P-value	0.36	0.48
BUN	R/Rs value	-0.08 (Rs)	-0.04 (R)
	P-value	0.63	0.85
Serum creatinine	R/Rs value	0.02 (R)	-0.19 (R)
	P-value	0.89	0.31
Serum Ca	R/Rs value	-0.12 (Rs)	0.13 (Rs)
	P-value	0.43	0.51
Serum Fe	R/Rs value	-0.09 (R)	-0.15 (Rs)
	P-value	0.57	0.45
H_2O_2	R/Rs value	0.16 (R)	-0.21 (Rs)
	P-value	0.30	0.29

Rs values were derived from Spearman's correlation analysis and R values from Pearson's correlation analysis. T1DM, type 1 diabetes mellitus; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; BMI, body mass index; AST, aspartate transaminase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; Ca, calcium; Fe, iron; H_2O_2, hydrogen peroxide; C-peptide, connecting peptide.

Table III. Correlation between C-peptide and cytokine concentrations in patients with T1DM and healthy volunteers.

	C-peptide		
Parameter	Correlation coefficient and P-value	T1DM	Healthy volunteers
IL-4	R/Rs value	-0.02 (Rs)	0.41 (Rs)
	P-value	0.92	0.03[a]
IL-5	R/Rs value	-0.01 (Rs)	-0.07 (Rs)
	P-value	0.94	0.79

[a]Statistically significant difference (P<0.05). Spearman's correlation analysis was used to analyze the correlations. T1DM, type 1 diabetes mellitus; C-peptide, connecting peptide.

In the present study, no significant correlations were observed between the C-peptide levels and all parameters measured, neither in patients with T1DM nor in HVs. Even the inverse correlations observed between the FBG and HbA1c levels with the C-peptide levels in patients with T1DM and those between H_2O_2 and C-peptide in the HVs, failed to yield statistically significant differences. The H_2O_2 levels were higher in patients with T1DM. However, they did not correlate with those of C-peptide. It has been reported that the enhanced glucose levels in patients with diabetes can lead to ROS generation. This process may be associated with the onset of diabetic vasculopathy and endothelial damage (16,26,27).

Several studies have been conducted with the aim of revealing the correlation between C-peptide levels and anthropometric (BMI, weight and pH), biochemical (FBG, HbA1c and adiponectin) and immunological (IL-2, IL-6, IL-10 and TNF-α) parameters in patients with recent-onset T1DM; these studies have revealed a strong and significant correlation between C-peptide levels and the aforementioned parameters (28-30). Additionally, statistically significant differences have been observed in the majority of the aforementioned measured parameters between patients with T1DM patients and controls (31).

C-peptide is an active biological peptide found in the circulation of healthy individuals and its levels are reduced or absent in patients with T1DM. C-peptide, as a peptide with anti-inflammatory activity, exerts beneficial effects. Therefore, its deficiency in individuals with T1DM may increase the risk of developing vascular complications (32,33). Furthermore, previous studies on the significance of persistent C-peptide levels in patients with T1DM have revealed that C-peptide levels are reduced in patients with prolonged disease and may not play a significant role in attenuating vascular complications (30,34). On the other hand, the pro-inflammatory effects of C-peptide and its deposits in vessel walls can lead to local inflammation, which is considered as a critical process in the development of atherosclerosis in patients with type 2 diabetes (35,36).

The present study also aimed to evaluate the correlations between the levels of C-peptide and those of cytokines. The serum levels of IL-4 significantly correlated with the C-peptide levels in the HVs. However, no significant correlation was observed between the levels of C-peptide, and those of IL-4 and IL-5 in the T1DM group. Therefore, the effects of anti-inflammatory cytokines in protecting β-cells warrant further investigation. A previous study demonstrated that the expression levels of both IL-4 and IL-5 were notably decreased in patients with T1DM (37). Additionally, other studies have indicated that IL-4 levels are downregulated in patients newly diagnosed with T1DM (38,39). Another study also indicated that IL-4 overexpression or the systematic administration of IL-4 could prevent the onset of insulitis and reduce the incidence of the disease (40). Herein, the IL-5 levels were decreased in patients with T1DM compared with the HVs. It has been reported that IL-4 and IL-5 can induce a regulatory phenotype, which promotes the secretion

of anti-inflammatory cytokines by immune cells (40). In a previous study, in an *in vivo* model of non-obese diabetic mice, IL-5 was shown to play a significant role in promoting the secretion of IL-10, which in turn exerted a potent suppressive effect on the development of T1DM (41). In addition, IL-5 has been shown to positively correlate with C-peptide in studies on the function of β-cells and the levels of secreted cytokines by peripheral blood mononuclear cells derived from patients withT1DM (37,42).

Herein, the majority of correlations were not significant, possibly due to the fact that the age range of the patients included was 7-25 years. These young patients could probably exhibit sufficient body physical mechanisms that react positively to the body's different vital activities. In addition, the enrolled patients with T1DM did not suffer from other diseases or other types of complications, while they had been diagnosed with the disease for >1 year.

The current study has certain limitations which should be mentioned. The main limitation of the study was that the sample size was small, since the samples were collected from only one center in the city of Karbala. In addition, only patients with T1DM with no pathological complications were enrolled. Finally, in some cases, the parents of children with T1DM did not provide consent for their children to participate in the study.

It was hypothesized that the anti-inflammatory effects of C-peptide may be compensated by other anti-inflammatory molecules in patients with T1DM. Therefore, the risk of pathological complications should not be excluded, due to high glucose-mediated H_2O_2, FBG and HbA1c, in addition to the reduced IL-5 levels.

In conclusion, the results revealed that the low concentrations of C-peptide in patients with T1DM were not associated with the measured parameters. However, these patients may still be at a high risk of developing pathological complications due to enhanced glucose and H_2O_2 levels, and decreased IL-5 levels.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SFH conceived the study. SFH, MBSAK and FAR performed the experiments. SFH and FAR helped to supervise the project. MBSAK and FAR contributed to sample preparation. All authors discussed the results and contributed to the final manuscript. SFH and FAR wrote the manuscript. SFH, MBSAK and FAR confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The protocol of the study followed the Declaration of Helsinki and was confirmed by the Council of the College of Science, University of Kerbala (Approval Code: no. 61793 on November 12, 2020). All participants filled in the consent form to participate in the study, and an agreement was obtained from the parents in the event that the participant's age was <16 years.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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