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Application of Circular Dichroism Spectropolarimetry for Discrimination between Type 2 Diabetic Patients and the Control Group

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ABSTRACT

It is well known that many biomolecules are chiral and their structure can be monitored by chiroptical spectroscopy. Nevertheless, their analysis in the natural environment of biofluids remains challenging. Recently, little efforts have been made to study biofluids by chiroptical techniques and find a specific signature for healthy/diseased conditions. Blood plasma can be a great subject for this line of examinations. In this study real clinical human blood plasma samples from 46 subjects were analyzed using CD spectropolarimetry to discriminate between type 2 diabetic patients and the control group. For more a challenging condition, patients were selected with type 2 diabetic and ulcer. The results showed a significant decrease in the total α-helical conformation of plasmatic proteins obtained from patients. Also total plasmatic protein content varied with disease condition. As a result, circular dichroism was useful to discriminate between the diabetic patients and the control group, and even between the diabetic patients with or without ulcer. Results of this study encourage possibility of using this technique as a useful supportive tool to conventional diagnostic methods.

Keywords: Biofluid, Human blood plasma, Chiroptical spectroscopy, Circular dichroism (CD), Type2 diabetes, Ulcer, Diagnosis

INTRODUCTION

Chirality- as a signature of life- and chiral elements starting with double strand DNA and L-amino acids as essential components of the human body, to non-coincident left and right human hands, shape up the world. As many biomolecules and potential biomarkers are chiral, their structure and behavior can be monitored by chiroptical spectroscopy [1,2].

Chiroptical techniques have great advantage of clarifying information about many biomolecules under controlled conditions with focus on pure substances or molecular systems in model environments [1,3-8]. So far, various studies on protein folding/misfolding under different experimental conditions [9-11]; determination of absolute configuration in pharmaceutical research [12-15] and the structure of essential biomolecules [1,16,17]; interactions between human serum albumin (HSA) and other proteins with drugs [18], cell membranes [19,20], *etc.* have been performed.

Natural environment and surrounding moieties may have a considerable influence on the structure and behavior of biomolecules. Therefore, study of essential biomolecules in biological fluids (biofluids) has a great potential to realize how biological processes occur. Despite of prevalent chiroptical studies by implementation to single molecules or simple mixtures, numerous efforts have been made to introduce chiroptical methods into biofluids analysis including blood plasma, urine, and vitreous [2,21-34].

Blood plasma which is a multi-functional biofluid, accounts for ~55% of total blood volume. It comprises more than 3000 individual proteins and peptides range from picogram to tens of milligrams per milliliter [35-37] whose biological function is determined by their structural characteristics [38]. It has been shown that some features of plasmatic proteins can be altered by some diseases such as

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their structure and concentration [39,40].

The preliminary application of circular dichroism spectropolarimetry as a chiroptical method to study blood plasma/serum was based on identification/kinetic study of a chiral agent such as drugs [21,29], cholesterol [24] or antibiotics [28,29] in blood plasma/serum after their intravenous or oral uptake. Some other studies were based on monitoring chiral agents such as flavonoids or polyphenols which bound to serum proteins such as HSA or globulins [41,42]. Another aim to study blood plasma with this methodology was finding a specific signature of its biomolecules which can provide information about healthy/diseased condition as a fast, minimally invasive and reagent-free method [30,32]. Some diseases have been analyzed with this aspect of study including colon cancer [31,43], pancreatic cancer [26], Alzheimer's disease [23], and type 1 diabetes mellitus [44].

Diabetes mellitus is apparently one of the oldest known human diseases base on the evidence in an Egyptian manuscript about 3000 years ago [45]. Diabetes mellitus spread all over the world increasingly, making an intricate pervasive socioeconomic problem [46-48]. The estimation of diseased population whom will have diabetes mellitus is 552 million people by the year 2030 [49,50] and 629 million people by the year 2049 [48,51]. The major contributor to the total diabetes population is type 2 diabetes [48] with an estimation of 439 million people by the year 2030 [52]. This type of diabetes mellitus (previously known as non-insulin dependent diabetes mellitus) has some regular features such as insulin resistance, insulin deficiency, and hyperglycemia in which blood glucose is abnormally high [53-55]. This type of diabetes mellitus with insidious onset and late recognition make patients very vulnerable to various forms of both short- and long-term complications [56] involving diabetic nephropathy, retinopathy, neuropathy, cardiovascular diseases, kidney disease, blindness, diabetic foot ulcer, and lower limb amputations [53-55,57-63]. About 25 percent of diagnosed patients with type 2 diabetes already have microvascular complications so that they have suffered from the disease for more than five years at the time of diagnosis [64].

Based on the presence of chiral structures which are related to the plasmatic biomolecules, we selected circular dichroism (CD) spectropolarimetry as a method which is inherently sensitive to their 2D structures [1,65], for this pilot study. We presumed that CD has the potential for detection of stereochemical changes in plasmatic biomolecules relating to pathological processes that occur during a disease. According to the complications arising from type 2 diabetes mellitus, it can be a challenging case to test this presumption. Therefore, in this study, we examined real biofluids of human blood plasma by CD spectropolarimetry to monitor possibility of discrimination between type 2 diabetic patients and control group. For a more challenging issue, some studied patients were selected with type 2 diabetic and ulcer.

MATERIALS AND METHODS

Participants

46 volunteers were signed up from regeneration group of Academic Center for Education, Culture and Research (ACECR), Tehran, Iran. Patient's consent/written acceptance was gathered for all samples according to the declaration of Helsinki principles. The tested group comprised 30 patients suffering from type 2 diabetes mellitus (14 with ulcers and 16 without ulcers) and 16 healthy individuals. Type 2 diabetic patients with ulcer had disease background for more than ten years, stage I or II ulcers without necrotic tissue or infected cartilage who had no addiction to narcotics and did not use antidepressants during the past 6 months. It is noticeable that healthy individuals were selected with conditions similar to the patients with ulcers. All healthy and diseased participants gave their written informed consent to cooperate in this study. This project was approved by ACECR and the ethics committee of breast cancer institute of ACECR (ethics no. : ir.acecr.ibcrc.rec.1394.38).

Blood Plasma Samples

Blood samples were collected using heparin as an anticoagulant reagent. Plasma fractions were then collected by centrifuging blood samples at 1500 × g for 10 min, which were frozen immediately (-20 °C).

Frozen plasma samples were thawed and dialyzed (MWCO 3,500 Da, Snake Skin Dialysis tubing, Thermo Fisher Scientific) for 24 h at 4 °C against 10 mM potassium phosphate, 150 mM sodium chloride, 15 mM sodium

citrate, pH = 7.5 to ensure complete solvent exchange. Samples were then collected and filtered through a 0.22 μm filter (polyethersulfone, Germany) to remove any possible particulates (protein aggregates or residual blood cells). The aliquots were stored at -20 °C prior to study. The dialysis buffer was also filtered (0.22 μm) and used for all sample dilutions and as a reference solution for circular dichroism studies. Before each measurement, samples were thawed at room temperature and were then diluted with buffer to a suitable concentration. Total protein concentration of plasma samples were assayed using Bicinchoninic Acid protein Assay method (BCA kit, purchased from sigma). Absorbance was measured at 562 nm and bovine serum albumin (BSA in BCA kit, purchased from sigma) was used as a reference protein.

Basically, BCA working reagent contains copper ion(II) which can bind to the protein at alkaline pH. Some elements in protein can result in the reaction of Cu^{2+} reducing to Cu^{+}. Binding of BCA to Cu^{+} causes a purple-blue complex to form [66]. These elements include cysteine, cystine, tryptophan, tyrosine, and the peptide bond [67]. Noteworthy unlike Bradford assay which is sensitive to the population of amine moieties, BCA method does not alter by non-enzymatic glycation of amino residuals in hyperglycemic conditions in diabetes mellitus. Consequently BCA method does not under estimate protein content. Thus, absorbance at 562 nm (which is correlated to the presence of purple-blue complex) will determine protein concentration.

Circular Dichroism (CD) Spectropolarimetry

CD signal is based on "left-handed absorption minus right-handed absorption" of circularly polarized photons (A_L - A_R) by a chiral element which is the basis of CD sensitivity to 2D structures [65]. The CD measurements were recorded using the J715 spectropolarimeter (Jasco, Japan) in two strategies and then analyzed with J715-Standard Analysis software. First, all filtered plasma similarly were diluted 200-times with filtered phosphate buffer (10 mM potassium phosphate, 150 mM sodium chloride, 15 mM sodium citrate, pH = 7.5). In the second strategy, plasma samples were diluted with different dilution factors to make 0.2 mg ml^{-1} concentration of total protein. After that far-ultra-violet CD measurements (185-250 nm) were carried out in a quartz cuvette with light path of 0.1 cm. The following parameters were used for measurements: resolution: 1 nm, sensitivity: 200 mdeg, scan speed: 100 nm min^{-1}, band width: 1.0 nm, response: 1 s at room temperature. Each measurement was repeated twice. All data were presented as mean ± SD and statistical evaluation was done by one sample Kolmogorov-Smirnov test at first to examine the normal distribution of data. For comparison of groups, analysis of variance (ANOVA) and a multiple comparison test of LSD (Least Significant Differences) as a post hoc test were used. $P < 0.05$ was considered significant. Statistical analysis was performed by SPSS version 16.0 software (SPSS Inc., Chicago,IL,USA).

RESULTS AND DISCUSSION

The Far-ultraviolet circular dichroism (far-UV CD) spectra of different human plasma samples with the strategies of same dilution factor and same total protein concentration are shown in Figs. 1 and 4, respectively. The average far-UV CD spectral patterns (Figs. 2 and 5) showed three distinct bands. Observed CD signals in this region is principally due to the presence of peptide bond (1). A weak broad negative band (centered around 209 nm) and a more intense positive band (around 190 nm) result from π-π* electron transitions. The π-electrons of the carbonyl groups play an important role in these transitions. The other weak broad negative band (around 222 nm) is resulting from n-π* electron transitions. Non-bonding electrons of oxygen in carbonyl groups make appearance of this band (region of 190-220 nm) (1,68-70). The shape and intensity of these bands depend on Φ and Ψ torsion angles in protein's backbone geometry (68,71). In this case, the peak resembled to those in α-helix-rich proteins, *i.e.* two partially overlapping negative bands at ~209 and 222 nm (1,32,70,71). Although human serum albumin is the most abundant α-helix-rich protein in plasma (~60% of total protein: 34-47 g l^{-1} of albumin per 70-75 g l^{-1} of total proteins), other plasmatic proteins and peptides are also partially responsible [72].

Plasma CD spectra of different people are shown in Fig. 1 with the strategy of using same dilution factor which are categorized in three groups as healthy individuals, type 2 diabetic patients without ulcer, and type 2 diabetic patients with ulcer. Their differences are clear in the average

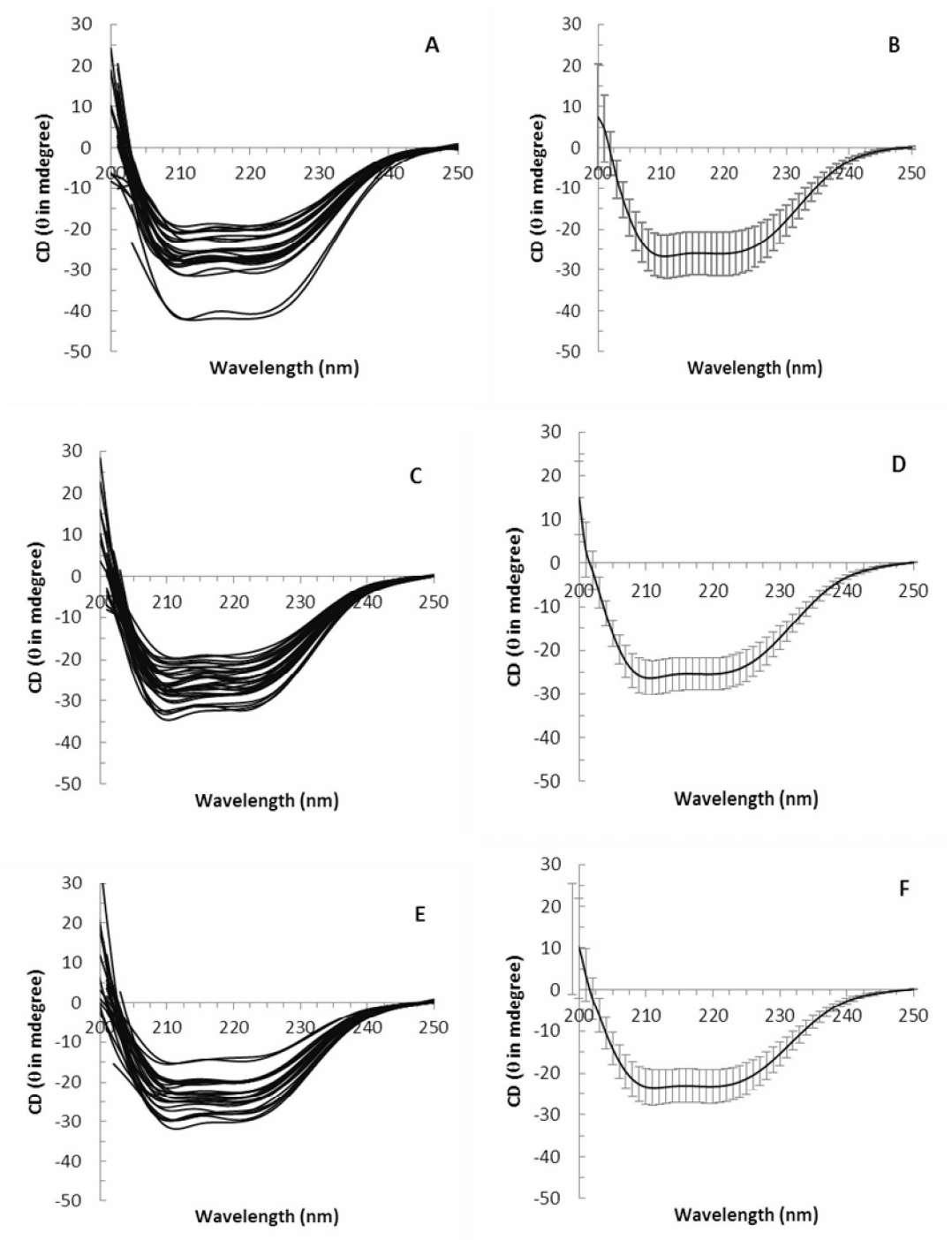

Fig. 1. Far-UV circular dichroism spectra of different human plasma samples with the same dilution factor of 1:200 (10 mM potassium phosphate, 150 mM sodium chloride, 15 mM sodium citrate, pH = 7.5). A and B: spectra from healthy individuals and their average spectrum respectively; C and D: spectra from type 2 diabetic patients without ulcer and their average spectrum respectively; E and F: spectra from type 2 diabetic patients with ulcer and their average spectrum respectively.

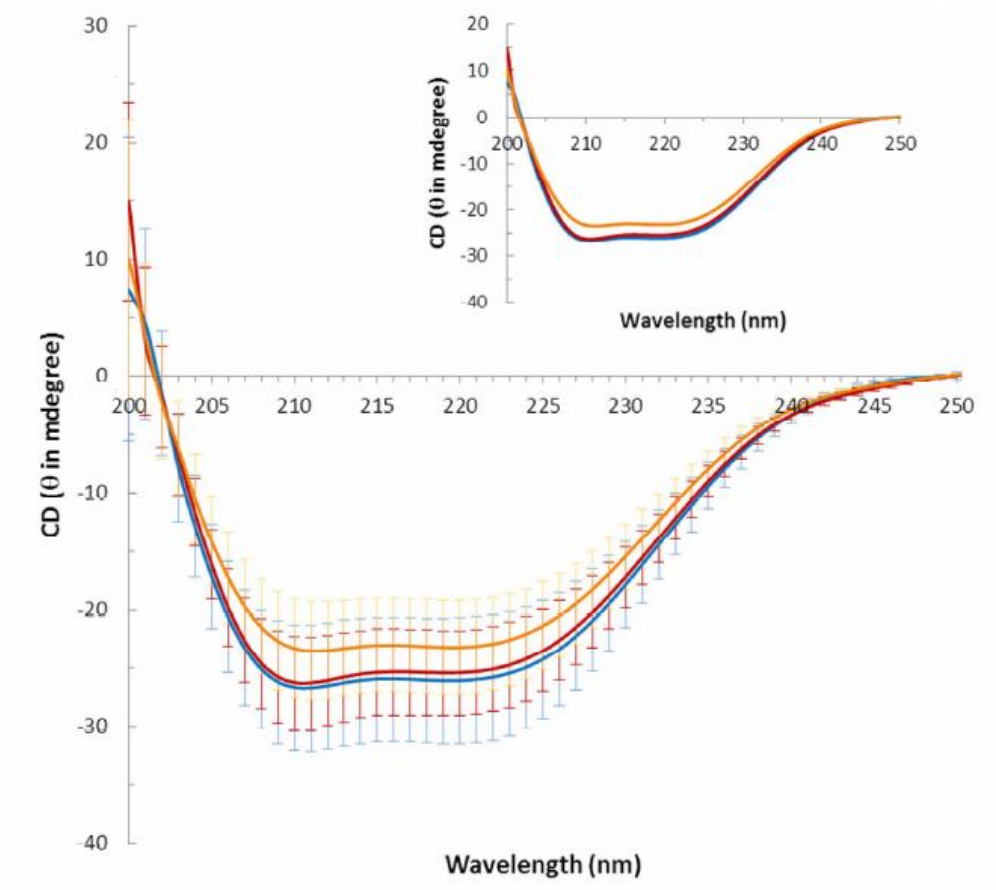

Fig. 2. Average far-UV circular dichroism spectra of different human plasma samples with the same dilution factor of 1:200 (10 mM potassium phosphate, 150 mM sodium chloride, 15 mM sodium citrate, pH = 7.5). Blue line: average spectrum of healthy individuals; Red line: average spectrum of type 2 diabetic patients without ulcer; Orange line: average spectrum of type 2 diabetic patients with ulcer. The inset is the same spectra without their error bars for further clarification.

spectra (Fig. 2). Although it seems that type 2 diabetic patients with ulcer group shows lower intensity, statistical analysis (Table 1) doesn't reveal significant difference among groups. In fact, multiple comparison of LSD in α-helicity index of θ_{222} showed: Firstly, there is no significant difference between healthy individuals and type 2 diabetic patients without ulcer (P = 0.666). Secondly, there is no significant difference between healthy individuals and type 2 diabetic patients with ulcer (P = 0.090). Thirdly, there is no significant difference between type 2 diabetic patients without ulcer and type 2 diabetic patients with ulcer (P = 0.189). Figure 3 shows a better comprehension of the mean groups variations.

Reduction in CD intensity due to some disease such as pancreatic cancer [26], Alzheimer's disease [23], and type1 diabetes mellitus [25], colon cancer [31] have been reported previously. Reduction in later case was more significant. Two main causes have been discussed for decrease in CD intensities: 1) reduction in values of the total plasma protein with no change in their native chiral structure, and 2) unfolding/cleavage of plasma proteins to achiral structures and subsequently increment of positive inflammatory proteins in order to maintain normal levels of other plasmatic proteins. The latter one was consistent with the

Table 1. The Statistical Results of ANOVA Test for θ_{222} of Circular Dichroism Measurements Resulted from the same Dilution Factor (1:200) of Different Human Plasma Samples. Healthy Individuals Group is Indicated as Class 1, Type 2 Diabetic Patients without Ulcer Group is Indicated as Class 2, and Type 2 Diabetic Patients with Group is Indicated as Class 3

θ_{222} For the same dilution factor of 1:200	Class 1 Mean ± SD	Class 2 Mean ± SD	Class 3 Mean ± SD	Test	P-value
	-25.79 ± 5.45	-25.10 ± 3.69	-22.92 ± 4.08	ANOVA	0.2081

LSD multiple comparisons showed: classes 1 & 2: NS* (P = 0.666); classes 1 & 3: NS (P = 0.090); classes 2 & 3: NS (P = 0.189). *NS means non-significant.

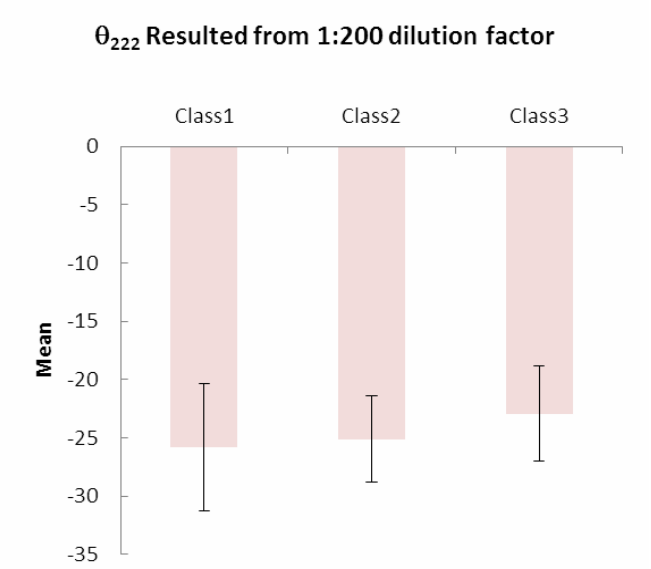

Fig. 3. Graphical presentation of average θ_{222} in each group of human plasma samples resulted from same dilution factor (1:200). Healthy individuals group is indicated as class 1, type 2 diabetic patients without ulcer group is indicated as class 2, and type 2 diabetic patients with ulcer group is indicated as class3.

pathophysiology of the case of type 1 diabetes mellitus [25,73,74].

In order to examine the difference of plasma protein contents in this case study, total protein concentration of studied groups was assayed using BCA method (Table 2). The results of BCA assay showed that there is a significant difference in the plasmatic protein content between the healthy individuals and type 2 diabetic patients without ulcer (with mean of 47.95 ± 11.08 and 55.58 ± 9.02, respectively and P = 0.047). Also, there is significant difference between healthy individuals and type 2 diabetic patients with ulcer (with mean of 47.95 ± 11.08 and 56.98 ± 11.46 and P = 0.024). Nevertheless there is no significant difference between type 2 diabetic patients without ulcer and type 2 diabetic patients with ulcer (P = 0.717). Therefore, in comparison with healthy

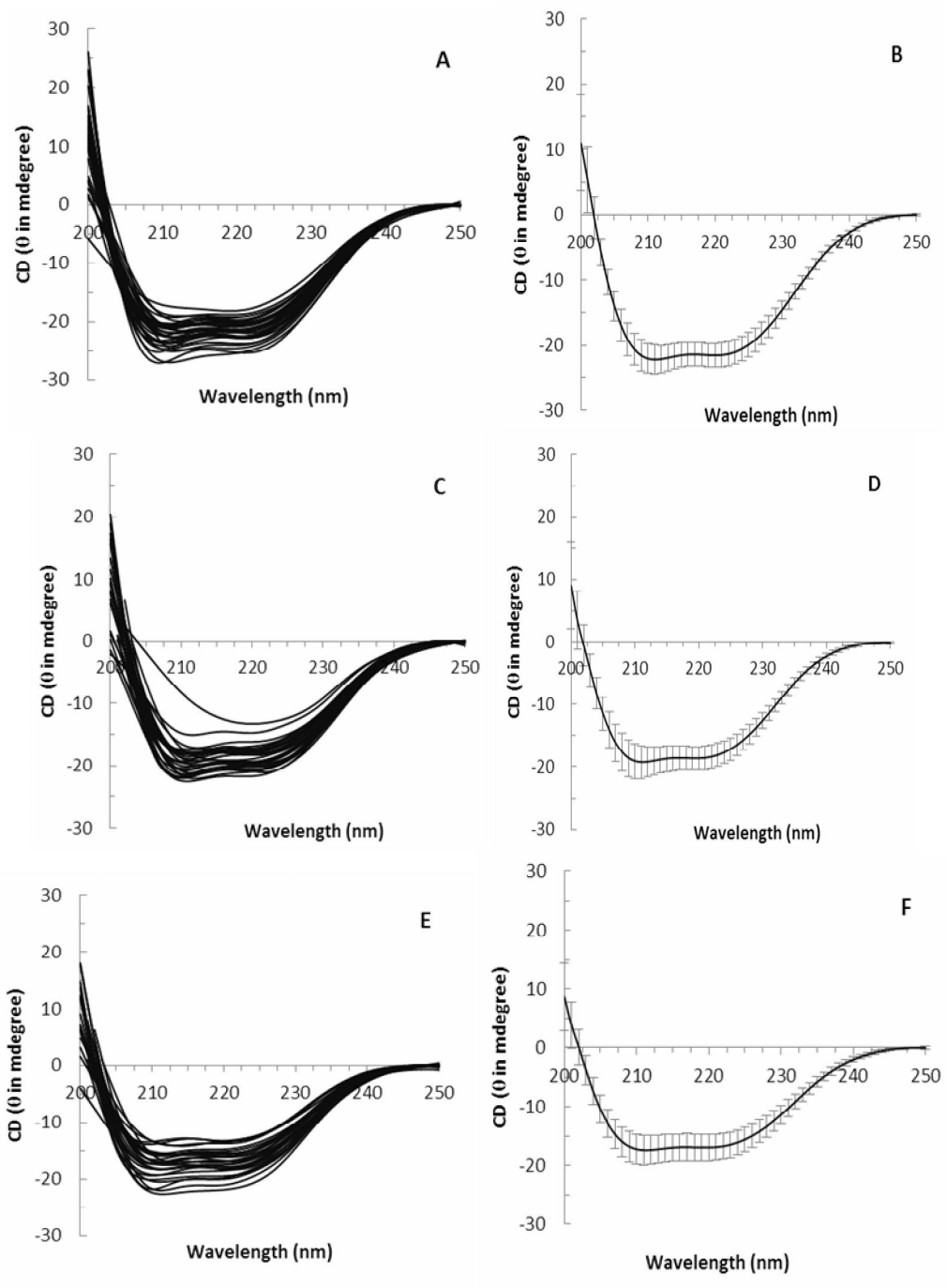

Fig. 4. Far-UV circular dichroism spectra of different human plasma samples with 0.2 mg ml^{-1} concentration of total protein (10 mM potassium phosphate, 150 mM sodium chloride, 15 mM sodium citrate, pH = 7.5). A and B: spectra from healthy individuals and their average spectrum respectively; C and D: spectra from type 2 diabetic patients without ulcer and their average spectrum respectively; E and F: spectra from type 2 diabetic patients with ulcer and their average spectrum respectively.

Table 2. The Statistical Results of ANOVA Test for Plasma Protein Contents Using BCA Method Resulted from Different Human Plasma Samples. Healthy Individuals Group is Indicated as Class 1, Type 2 Diabetic Patients without Ulcer Group is Indicated as Class 2, and Type 2 Diabetic Patients with Ulcer Group is Indicated as Class 3

Plasma protein contents using BCA method (mg ml^{-1})	Class 1 Mean ± SD	Class 2 Mean ± SD	Class 3 Mean ± SD	Test	P-value
	47.95 ± 11.08	55.58 ± 9.02	56.98 ± 11.46	ANOVA	0.047

LSD multiple comparisons showed: Classes 1 & 2: SN* (P = 0.047); classes 1 & 3: SN (P = 0.024); classes 2 & 3: NS* (P = 0.717). *SN means significant. *NS means non-significant.

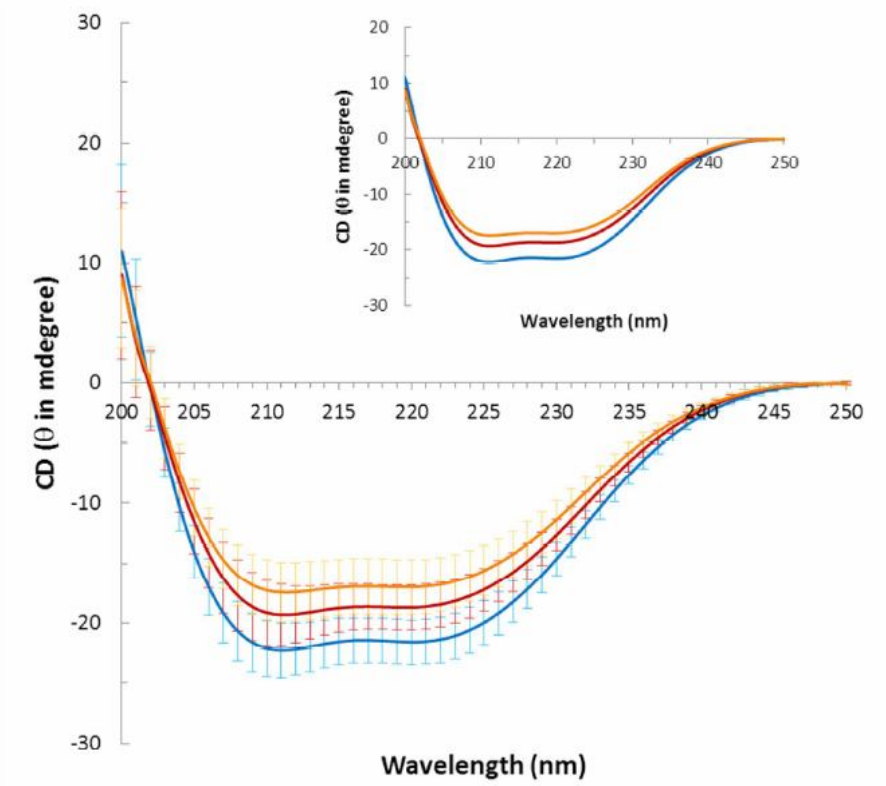

Fig. 5. Average far-UV circular dichroism spectra of different human plasma samples with 0.2 mg ml^{-1} concentration of total protein (10 mM potassium phosphate, 150 mM sodium chloride, 15 mM sodium citrate, pH = 7.5). Blue line: average spectrum of healthy individuals; Red line: average spectrum of type 2 diabetic patients without ulcer; Orange line: average spectrum of type 2 diabetic patients with ulcer. The inset is the same spectra without their error bars for further clarification.

individuals, total protein content of type 2 diabetic patients were increased.

Plasma CD spectra of different people are shown in Fig. 4 with the strategy using the same total protein concentration (0.2 mg ml^{-1}) which are categorized in three groups or classes as: 1) healthy individuals, 2) type 2

Table 3. The Statistical Results of ANOVA Test for θ_{222} of Circular Dichroism Measurements Resulted from the Same Total Protein Concentration (0.2 mg ml^{-1}) of Different Human Plasma Samples. Healthy Individuals Group is Indicated as Class 1, Type 2 Diabetic Patients without Ulcer Group is Indicated as Class 2, and Type 2 Diabetic Patients with Ulcer Group is Indicated as Class 3

θ_{222} For the same total protein concentration (2 mg ml^{-1})	Class 1 Mean ± SD	Class 2 Mean ± SD	Class 3 Mean ± SD	Test	P-value
	-21.27 ± 1.79	-18.44 ± 1.68	-16.76 ± 2.01	ANOVA	0.001

LSD multiple comparisons showed: classes 1 & 2: SN* ($P < 0.001$); classes 1 & 3: SN ($P < 0.001$); classes 2 & 3: SN ($P = 0.010$). *SN means significant.

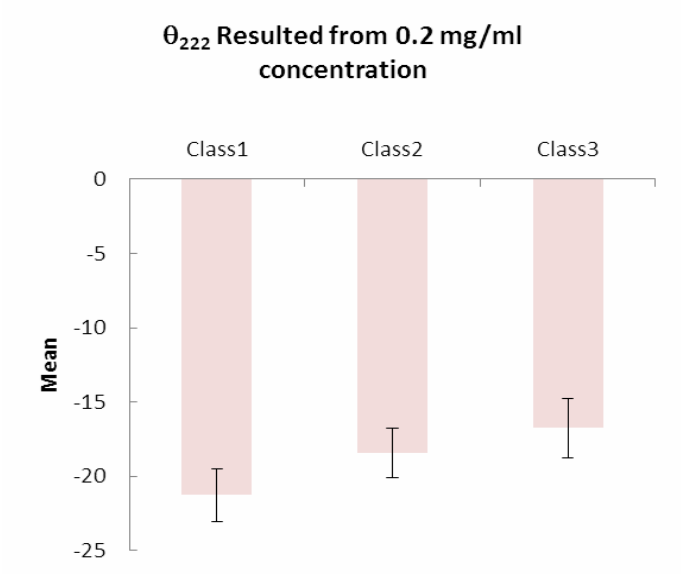

Fig. 6. Graphical presentation of average θ_{222} in each group of human plasma samples resulted from same total protein concentration (0.2 mg ml^{-1}). Healthy individuals group is indicated as class 1, type 2 diabetic patients without ulcer group is indicated as class 2, and type 2 diabetic patients with ulcer group is indicated as class 3.

diabetic patients without ulcer, and 3) type 2 diabetic patients with ulcer. It should be noted that this approach is more reliable because there is no exact molecular weight for total plasma components in healthy and unhealthy conditions to calculate molar ellipticity from CD signals. Differences among groups are clear in the average spectra (Fig. 5). Using the same total protein concentration not only shows meaningful differences among healthy individuals and type 2 diabetic patients, but it also can differentiate between patients with or without ulcer (Table 3). Indeed, LSD multiple comparison showed: Firstly, there is significant difference between healthy individuals and type 2 diabetic patients without ulcer in α-helicity index of θ_{222} ($P < 0.001$). Secondly, there is significant difference between healthy individuals and type 2 diabetic patients with ulcer in α-helicity index of θ_{222} ($P < 0.001$). Thirdly, there is a significant difference between type 2 diabetic patients without ulcer and type 2 diabetic patients with

ulcer in α-helicity index of θ_{222} (P = 0.015). Therefore, all studied groups are significantly different with each other, in which, CD signals shows lower intensity in patients and even less intensity in acute condition (patients with ulcer). See Fig. 6 for better comprehension of the mean groups' variations.

In the second strategy, because of using the same total protein concentration, the difference in CD signals between the studied groups did not origin from the differences between total protein content. Thus, the lower intensity of the patient spectrum may correlate with higher content of the less ordered structures such as partial misfolding of albumin and other α-helical peptides/proteins during the development of diabetic-related pathological states. On the other hand, using the same total protein content can lead to a better discrimination between the diseased individuals and the control group. Therefore, circular dichroism had a useful potential to discriminate between the diabetic patients and the control group and even between the diabetic patients with or without ulcer.

CONCLUSIONS

Real clinical human blood plasma samples from 46 subjects (type 2 diabetic patients and healthy controls) were analyzed using CD spectropolarimetry for monitoring the overall skeletal conformation of plasmatic chiral elements. The results showed a significant decrease in the α-helical conformation of plasmatic proteins obtained from patients. This noticeable finding resulted from the strategy of using the same total protein concentration (0.2 mg ml^{-1}). Based on our observations not only the total protein content, but also the secondary structure elements vary with disease development. Therefore, in this pilot study, we successfully identified that definite conformational differences exist between the diabetic patients and the control group and even between the diabetic patients with or without ulcer. In fact, according to our study and the previous findings, results of this study encourage possibility of using this technique as a useful supportive tool to conventional diagnostic methods.

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