Hemolysis Affects C-Peptide Immunoassay

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Background: C-peptide is used widely as a marker of insulin secretion, and it participates in the inflammatory response and contributes to the development of coronary artery disease (CAD) in patients with type 2 diabetes mellitus (T2DM). Previous studies have reported that C-peptide measurement was unaffected by hemolysis. However, we found that hemolysis negatively affected C-peptide assay in routine laboratory practice. We further established and validated an individualized hemolysis correction equation to correct and report accurate serum C-peptide results for hemolyzed samples. **Methods:** We studied the effects of hemolysis on C-peptide assay by adding lysed self red blood cells (self-RBCs) to serum. An individualized correction equation was derived. Further, we evaluated the performance of this individualized correction

equation by artificially hemolyzed samples. **Results:** C-peptide concentration decreased with increasing degree and exposure time of hemolysis. The individualized hemolysis correction equation derived: $C-P_{corr} = C-P_{meas} / (0.969 - 1.5Hb_{serum/plasma} - 5.394 \times 10^{-5} \text{ Time})$, which can correct bias in C-peptide measurement caused by hemolysis. **Conclusions:** Hemolysis negatively affects C-peptide measurement. We can correct and report accurate serum C-peptide results for a wide range of degrees of sample hemolysis by individualized hemolysis correction equation for C-peptide assay. This correction would improve diagnostic accuracy and reduce inappropriate therapeutic decisions. *J. Clin. Lab. Anal.* **30**:1232–1235, 2016. © 2016 Wiley Periodicals, Inc.

Key words: C-peptide; hemolysis; hypoglycemia; individualized correction

INTRODUCTION

C-peptide is used widely as a marker of insulin secretion, and it participates in the inflammatory response and contributes to the development of coronary artery disease (CAD) in patients with type 2 diabetes mellitus (T2DM) (1–4). Hemolysis is a primary preanalytical problem in laboratory diagnostics, with a frequency of 3% of all diagnostic specimens (5,6). It has been documented that hemolysis causes severe negative interference on insulin assay due to RBC insulin-degrading enzyme (IDE)(7–10). Meanwhile, some studies have reported that C-peptide measure was unaffected by hemolysis (10,11). However, we found that hemolysis also resulted in a negative interference on C-peptide assay in routine laboratory practice, and the effect of hemolysis on C-peptide assay was similar but weaker than that reported in insulin assay. Here, we demonstrated that the percent of change in concentration of C-peptide, as a result of the hemolysate spikes, was associated with percentage

and exposure time of hemolysis. We derived and validated an individualized hemolysis correction equation in an attempt to correct and report accurate serum C-peptide results for a wide range of degrees of sample hemolysis.

MATERIALS AND METHODS

Study design

This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. This study consisted of (a) ascertaining the

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effect of hemolysis on C-peptide assay, (b) generating an individualized correction equation accounting for hemolysis effect, and (c) measuring C-peptide in human samples before and after inducing in vitro hemolysis, to evaluate the performance of individualized hemolysis corrections.

Experimental subjects and samples

For the determination of the effect of hemolysis on C-peptide assay, we collected 13 serum samples (free of hemolysis) with C-peptide concentrations of 2080–5408 pmol/l and whole-blood samples with K₂EDTA from 13 donors. To evaluate the individualized correction equation, we collected 33 samples from another 11 donors. Each donor was collected three samples, including two serum samples and one sample anticoagulated with K₂EDTA.

Preparation of hemolysates

We centrifuged EDTA whole-blood specimens at $1100 \times g$ for 10 min, removed the plasma, and washed the packed RBCs five times to remove any extracellular C-peptide. Then, the RBCs were resuspended in normal saline, the volume being equal to that of plasma removed. Hemolysis was then induced by using the osmotic shock method. After freezing at -20°C , the sample was thawed, mixed, and centrifuged. The hemolysates were transferred to a clean tube, and the hemoglobin concentration was measured. According to the ratio of hemoglobin concentration in normal saline to that in plasma, we got the percentage of hemolysis. Then, the hemolysates were added to serum to obtain the percentage of hemolysis of 10, 5, 2.5, 1.25, and 0.625, respectively.

Quantitation of C-peptide

C-peptide was measured using a sandwich immunoassay with the electrochemiluminescence (ECL) technology installed on a Modular E602® (Roche Diagnostics, Mannheim, Germany). The method has an internally validated interassay CV of <2.3% across the analytical measurement range (AMR) of 3–13300 pmol/l.

Quantitation of hemoglobin

Hemoglobin concentration of the whole-blood sample was measured by the Sysmex XE-2100 hematology analyzer. Free hemoglobin concentration in serum was analyzed by the type 721 spectrophotometer (Lengguang Technology, Shanghai, China) as described in more detail elsewhere (12). Absorbance was measured at 435 nm.

Derivation of an individualized hemolysis correction equation

Thirteen serum pools with various starting C-peptide concentrations (2080–5408 pmol/l) were prepared by combining residual serum samples. Each serum pool, with a known baseline C-peptide concentration, was divided into five aliquots. The hemolysates were added to serum to obtain the percentage of hemolysis of 10, 5, 2.5, 1.25, and 0.625, respectively. Meantime, a serum pool added by normal salt equal volume to the hemolysates was used as a control. These samples were incubated at room temperature for 0 (immediately after hemolysates added), 1, 2, 4, and 8 hr, and C-peptide concentrations were measured.

The relationship between percent of change in concentration of C-peptide and percentage and exposure time of hemolysis was determined. The change in C-peptide concentration in the serum pools was measured and plotted as a function of hemolysate. The least squares linear fit was determined for percent of change in concentration of C-peptide and plotted as a function of percentage and exposure time of hemolysis.

Performance evaluation of the individualized correction equation

We collected 33 samples from 11 donors. Each donor was collected three samples, two serum samples, and one sample anticoagulated with K₂EDTA. Two serum samples were used to induce hemolysis and measure the original C-peptide concentration, respectively. The anticoagulated whole-blood sample was used to calculate the percentage of hemolysis. The C-peptide concentration of hemolyzed sample and percentage and exposure time of hemolysis were calculated in the correction equation. This generated a corrected C-peptide result that was compared with the original C-peptide measured in the non-hemolyzed baseline serum sample.

Statistical analysis

Statistical analysis was performed with SPSS 16.0. Two-tailed *t*-test was used for significance testing between groups of continuous data, if necessary after data transformation. *P* value <0.05 was considered statistically significant.

RESULTS

We studied the effects of hemolysis on C-peptide assay by adding lysed self-RBCs to serum. The hemolysates were added to serum to obtain the

percentage of hemolysis of 10, 5, 2.5, 1.25, and 0.625. These samples were incubated at room temperature for 0 (immediately after hemolyzates added), 1, 2, 4 and 8 hr, and then C-peptide concentration were measured. The results showed that C-peptide concentration decreased with increasing percentage and exposure time of hemolysis in all pools (Fig. 1). Our data showed that after normalizing the measured C-peptide in each pool to the respective initial starting concentration and then plotting the change as a function of increasing percentage and exposure time of hemolysis, the least squares linear fit was determined for percent of change in concentration of C-peptide (% Δ C-P) and plotted as a function of percentage and exposure time of hemolysis: % Δ C-P = $1.5\text{Hb}_{\text{serum/plasma}} + 5.394 \times 10^{-5} \text{Time} - 0.031$ with an $R^2 = 0.953$. Based on this, we further derived an individualized correction equation: $\text{C-P}_{\text{corr}} = \text{C-P}_{\text{meas}} / (0.969 - 1.5\text{Hb}_{\text{serum/plasma}} - 5.394 \times 10^{-5} \text{Time})$. This equation provides the corrected C-peptide concentration (C-P_{corr}, pmol/l) based on the measured serum C-peptide concentration (C-P_{meas}, pmol/l), exposure time of hemolysis (min), the concentration of free Hb in the serum (Hb_{serum}, mg/dl), and the concentration of Hb (Hb_{plasma}, mg/dl) for an individual, which is normalized to percentage of hemolysis.

In order to evaluate the individualized correction equation, we collected 33 samples from 11 donors. Each donor was collected three samples, including two serum samples and one sample anticoagulated with K₂EDTA. Two serum samples were used to induce hemolysis and measure the original C-peptide concentration, respectively. The EDTA whole-blood sample was used to calculate percentage of hemolysis. The C-peptide concentration of hemolyzed sample and

percentage and exposure time of hemolysis were entered into the correction equation. This generated a corrected C-peptide result that was comparable with the original C-peptide measured in the non-hemolyzed baseline serum sample. The results showed that the individualized correction equation reverted all C-peptide concentrations of the hemolyzed samples to values that were statistically not different from the baseline measurements ($P = 0.3058$) (Fig. 2). These results suggested that the individualized hemolysis correction equation was able to correct serum C-peptide results for a wide range of degrees of sample hemolysis.

DISCUSSION

This study shows that hemolysis can negatively interfere with C-peptide assay in clinical laboratory practice, and this effect increased with the degree and exposure time of hemolysis. Our data showed that the decrease in concentration of C-peptide was less than 2% when samples were exposed to 2.5% hemolysis for 8 hours at room temperature. However, the C-peptide reduced more than 10%, when samples were exposed to 10% hemolysis only for 1 hr, which indicated high degrees of hemolysis affected C-peptide assay significantly and should not be ignored in clinical diagnosis and therapeutic decisions.

Previous studies have reported that hemolysis unaffected C-peptide assay (10,11), which is in contradiction to our results. We think that this should be caused by the difference in treatment of hemolyzed samples between previous studies and ours. The hemolyzed samples were separated immediately and stored at -20°C before C-peptide assay in previous studies (11). However, we placed the hemolyzed samples at room temperature from 0 to 8 hr before C-peptide assay, which was in line with routine clinical

Fig. 1. Effect of percentage and exposure time of hemolysis on percent of change in concentration of C-peptide (% Δ C-P).

Fig. 2. Correction of decreased C-peptide concentrations resulting from intentional hemolysis compared to baseline. Serum C-peptide concentrations (pmol/l) of baseline and artificially hemolyzed specimens collected from 11 donors were plotted along with the corrected C-peptide value obtained using the individualized equations. Triple asterisk indicates $P < 0.001$. "NS" indicates no significance.

laboratory practice. Although the mechanism by which the measured C-peptide is reduced was uncertain, we speculated it should be similar to that of insulin, considering that the key factors of decrease in C-peptide, such as percentage and exposure time of hemolysis, were the same as in insulin. In this regard, our experimental conditions could truly show the effect of hemolysis on C-peptide assay in routine clinical laboratory practice.

Based on the measured serum C-peptide concentration ($C-P_{\text{meas}}$, pmol/l), exposure time of hemolysis (min), the concentration of free Hb in the serum (Hb_{serum} , mg/dl), and the concentration of Hb (Hb_{plasma} , mg/dl) for an individual, we derived an individualized hemolysis correction equation for the determination of C-peptide in hemolyzed samples. To evaluate the individualized correction equation, we collected another 33 samples from 11 donors. The C-peptide concentration of hemolyzed sample and percentage and exposure time of hemolysis was entered into the correction equation. This generated corrected C-peptide results that were comparable with the original C-peptide measured in the non-hemolyzed baseline serum sample. The results showed that the individualized correction equation reverted all C-peptide concentrations of the hemolyzed samples to values that were statistically not different from the baseline measurements, which indicated that accurate serum C-peptide results could be reported across a wide range of degrees of sample hemolysis by individualized hemolysis correction equation for C-peptide assay. The major limitation to incorporating this individualized correction is that it requires to collect a whole-blood sample or information of the concentration of Hb_{plasma} by which calibrate the percentage of hemolysis. In addition, the amount of samples in our study is small, and this correction equation need to be further verified with more clinical samples.

In conclusion, we first demonstrate that hemolysis can affect C-peptide assay, and we derive and validate an individualized hemolysis correction equation for the determination of C-peptide in different degree of hemolyzed samples. This correction would improve diagnostic accuracy and reduce inappropriate therapeutic decisions.

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