Clin. Lab. 2021;67:XXX-XXX
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ORIGINAL ARTICLE

Evaluation of the Effect of Hemolysis on Quantitative Chemiluminescent Immunoassay Results for 10 Analytes

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SUMMARY

Background: **The aim is to evaluate the effect of hemolysis on the quantitative chemiluminescent immunoassay results of 10 analytes and to provide a basis for formulating specific sample rejection criteria and reviewing report results.**
Methods: **Hemolysis based on the clinical hemolysis index, hemolysis 1+, 2+, and 3+ samples and matched normal samples were collected. The quantitative chemiluminescent immunoassay results of 10 analytes from the two samples (hemolysis and normal) were determined and differences between the results obtained from samples with different degrees of hemolysis and those obtained from normal samples were evaluated.**
Results: **A total of 34 pairs of samples were collected, including 10 pairs of 1+ hemolysis samples, 10 pairs of 2+ hemolysis samples, and 14 pairs of 3+ hemolysis samples. The quantitative chemiluminescence immunoassay detection results for the 10 analytes showed that regardless of the degree of hemolysis, the differences in alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen (CA19-9), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and ferritin (FER) between the hemolysis and normal samples were all lower than the total allowable error (TEa) based on biological variation; there were no statistically significant differences between the samples. However, the results for insulin (INS) began to decrease significantly at a hemolytic index of 1+, folic acid (FOL) showed an increase at a hemolytic index of 2+, and there was a significant difference at a hemolytic index of 3+.**
Conclusions: **This research identified the analytes that are susceptible to hemolysis interference in chemiluminescent immunoassays. The influence of hemolysis on hemolytic clinical laboratory tests was closely related to the assay system used; thus, laboratories should evaluate the effect of hemolysis on their own analysis systems and define assay-specific hemolysis warning indices.**
(Clin. Lab. 2021;67:xx-xx. DOI: 10.7754/Clin.Lab.2021.210150)

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Manuscript accepted March 30, 2021

KEY WORDS

hemolysis, chemiluminescent immunoassay, evaluation studies

INTRODUCTION

Clinical laboratory medicine quality control can be classified as pre-analysis, analysis, and post-analysis. Hemolysis is a type of pre-analysis quality control issue. It is the most common problem in the pre-treatment of specimens, with an incidence of up to 3.3% [1], and accounts for 60 - 70% of all unqualified specimens in our laboratory's quality index statistics. Hemolysis usually

occurs when the blood is drawn roughly, the specimen is transported incorrectly or the centrifugal velocity is improper. Hemolysis can have a strong influence on the results of sample analyses [2]. Currently, an increasing number of laboratories are establishing biochemical immune assembly lines and use the serum hemolysis index (HI) to quickly and accurately identify hemolytic samples. The HI of a biochemical instrument is determined by mixing serum with saline; the absorbance of hemoglobin in the sample at a wavelength of around 410 nm provides a semi-quantitative determination of the hemoglobin (Hb) concentration. 1+ indicates that the concentration of Hb > 30 mg/L, 2+ indicates that the concentration of Hb > 100 mg/L, and 3+ indicates that the concentration of Hb > 500 mg/L. The hemolysis HI can be used to alert laboratory technicians to the severity of hemolysis in a specimen. However, a common question is, how much does the degree of hemolysis degree affect the test results of different items? How much influence does it have on the immunoluminescence projects such as tumors and hormones, which use antigen and antibody combination as the detection principle? When testing such items, it may be difficult to draw blood on some elderly people or infants leading to hemolysis. Do we need to return these specimens?

Different analysis systems have their own specific instructions, but it is necessary for technicians to verify the instructions so that each analyte can be evaluated in combination with the laboratory's own quality requirements; the data provided by the manufacturer may need to be adjusted according to the evaluation results. The effects of sample hemolysis on biochemical analytes have been widely studied [3-5]. Optical interference caused by various substances released into the serum after red blood cell rupture and chemical reactions with reagent components may interfere with the analyte concentration to varying degrees, resulting in falsely increased or decreased results for multiple analytes [6]. For quantitative chemiluminescent immunoassays, the antigen-antibody binding sites designed by different analyte systems differ and the effect of hemolysis on these analytes is diverse; thus, there is variation in the results of published studies. During the sample pre-treatment process for quality control, different degrees of sample hemolysis caused by endogenous and exogenous sources are inevitable; however, the interference from hemolysis may result in serious errors in laboratory results. Therefore, it is necessary to evaluate the influence of different degrees of hemolysis on the test results of different quantitative chemiluminescence immunoassay items based on the sample hemolysis index (HI) measured by the laboratory automatic biochemical analyzer and to develop the appropriate basis for specimen rejection.

MATERIALS AND METHODS

Sample collection

Residual serum classified as HI ≥ 1+ was obtained from clinical samples sent to the Department of Laboratory Medicine, the Affiliated People's Hospital of Fujian University of Traditional Chinese Medicine. Normal serum collected at the same time served as the control (the parallel samples were obtained from a multi-tube serum sample collected from the same patient). Hemolysis samples were classified as mild (1+), moderate (2+) or severe (3+) according to the HI. From May 2019 to September 2020, 34 pairs of samples were collected, among which 10 pairs were mild, 10 pairs were moderate, and 14 pairs were severe.

Measurement of samples

Five chemistry analytes were measured on an Abbott Architect i2000 (Abbott Laboratory, USA): alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA199), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). All reagents used were Abbott's original reagents. The Architect i2000 system uses chemiluminescent microparticle immunoassay (CMIA) technology. The microparticles are coated with capture molecules and then incubated with the analyte to form immune complexes. After separation of the unbound microparticles, the luminescent emission is measured.

Five chemistry analytes were measured on a Beckman Coulter DXI 800 (Beckman Instruments, USA): folic acid (FOL), ferritin (FER), vitamin B12 (Vit B12), cortisol (CORT), and insulin (INS). All reagents used were Beckman Coulter's original reagents. This assay utilizes CMIA technology, with a monoclonal item-specific ‘capture’ antibody and a ‘detection’ antibody labelled with alkaline phosphatase, which is also item-specific. Chemiluminescent substrate, Lumi-Phos 530, is added into the reaction tube and reacts with intrinsic factor-labelled alkaline phosphatase in the reaction system. The emitted light quantum is detected by a photomultiplier tube. The analyte intensity of the light quantum was calculated for each sample.

All analytes were subjected to daily internal quality control before the tests were performed; Bio-Rad quality control materials were used (Bio-Rad Laboratories, Inc., USA). Within 1 hour of sample collection, chemiluminescent immunoassays of the 10 analytes were simultaneously performed for both the normal and hemolyzed samples.

Calculations and Statistical analysis

The data were analyzed by SPSS 22.0 statistical software. For each analyte, paired *t*-tests were used to compare the hemolysis group and the normal control group. A p-value less than 0.05 indicated a statistically significant difference.

Bias% was calculated as follows: (hemolytic group value - normal group value)/normal group value 100%.

According to the quality evaluation requirements of the National Health Commission Clinical Laboratory Centre (NCCL) and the total allowable error based on biological variation (TEa), the quality evaluation requirement of the 10 analytes was 25%. Therefore, the TEa was uniformly adopted as the evaluation standard for Bias% in this study. The Bias% of each analyte was compared to the TEa and the hemolysis warning index of the analyte was determined after discussion between senior laboratory technicians and clinicians.

RESULTS

Effect of hemolysis on analytes

Table 1 lists the concentration of each analyte (mean ± SD) for the hemolysis group and normal group. The effect of hemolysis on the test results of the two groups is shown in Table 1.

The concentration of INS decreased with increasing hemolysis level when HI = 1+ hemolysis, while FOL began to significantly increase at HI = 1+ hemolysis. Further, the Vit B12 and CORT concentrations of the hemolysis group (3+) were increased significantly compared to the normal group. There were no significant differences in AFP, CEA, CA199, LH, FSH, and FER between the hemolysis group and normal group.

Determination of hemolysis warning index

As shown in Figure 1, when HI = 1, the Bias% of INS in the hemolysis group exceeded the TEa (> 25%); thus, the hemolysis warning level for this analyte was set as 1. When HI = 2+, the FOL measurement of the hemolysis group deviated more than the normal group based on TEa; thus, the hemolysis warning level for this analyte was set to 2. Although the concentrations of Vit B12 and CORT were significantly impacted when HI = 3+, the Bias% did not exceed the TEa, as shown in Figure 2; thus, the hemolysis warning level was set to 3. There were no significant differences in the concentrations of AFP, CEA, CA199, LH, FSH, and FER between the hemolysis and normal groups, so the hemolysis warning levels for these analytes were set to 4. The warning indices are presented in Table 2.

Comparison of various studies of hemolysis in immunoassays

A summary of studies on hemolysis in immunoassays is presented in Table 2. The findings in the previous literature for hemolytic interference in relation to INS, FOL, CORT, AFP, CEA, CA199, LH, and FSH were basically consistent with the findings of the current study. A previous study reported that hemolysis negatively interfered with Vit B12 which is in contrast to our findings. Our study showed that FER was increased, but there was no significant difference between the hemolysis and normal group, while other studies have reported positive interference from hemolysis.

DISCUSSION

This study evaluated the effects of hemolysis on quantitative chemiluminescent immunoassay results for our laboratory assay system. Hemolysis is a common preanalytical interference factor in clinical specimens [7]. *In vitro* hemolysis is caused by mechanical damage in the process of specimen collection, transportation, treatment, and/or preservation [8,9]. *In vivo* hemolysis can be hereditary or caused by acquired and iatrogenic diseases such as autoimmune hemolytic anemia, blood transfusion reactions, severe infection, and/or other diseases [10]. At present, most studies on the influence of hemolysis on detection results are focused on biochemical measurements. There are few studies of quantitative chemiluminescent immunoassay results. Hemolysis affects the detection results primarily in the following ways: (1) substances released by hemolysis rupture enter the serum and interfere with the detection results; (2) Hemolytic hemoglobin release causes interference in spectral detection; (3) substances released by hemolysis interact with substances to be measured, affecting the detection results [4]. Therefore, healthcare providers should pay close attention to the limitations of these assays. It is important to recognize the potential for interference in immunoassays and to set precautionary measures to identify this interference whenever possible. Detecting the presence of interference may require pretreatment of the sample before the actual analysis. The current study revealed that the concentrations of INS, FOL, CORT, and Vit B12 were significantly affected by hemolysis. INS exhibited significant negative interference when HI = 1+. As shown in Table 1, with the aggravation of hemolysis, the value of INS also gradually decreased. This result is consistent with reports in the literature [11-13]. After red blood cell rupture, INS degrading enzyme (IDE) is released. IDE can efficiently and specifically degrade INS, resulting in low results in hemolyzed samples [14,15]. The more severe the degree of hemolysis, the more IDE is released from red blood cells, the more INS is decomposed, and the more significant the INS interference. This interference can also result in an increase in FOL, as shown in Table 1. In the current study, the concentration of FOL gradually increased with an increasing degree of hemolysis. Because the concentration of FOL in erythrocytes is 16.7 times that in extracellular fluid, FOL in erythrocytes is released into the extracellular space when hemolysis occurs, which leads to an increase in FOL. The more severe the erythrocyte rupture, the more FOL is released, and the greater the impact on the assay. CORT was also increased in hemolysis samples. This increase was statistically significant in hemolysis 3+ samples. Hasanato R. et al. used an Abbott system and reported that CORT was increased by hemolysis [11]. Lucena R. et al. also found consistent results [16]. The possible reason for this finding is that substances released during erythrocyte rupture in hemolysis interfere with the antigen-antibody reaction, resulting in falsely increased re-

Table 1. Comparison of results between the hemolysis group and normal group.

Analyte	Unit	Hemolysis 1+			Hemolysis 2+			Hemolysis 3+		
		Normal (mean ± SD)	Hemolysis (mean ± SD)	p	Normal (mean ± SD)	Hemolysis (mean ± SD)	p	Normal (mean ± SD)	Hemolysis (mean ± SD)	p
INS	uIU/mL	10.10 + 7.43	6.80 + 5.1	0.003 *	15.62 ± 16.0	5.39 ± 6.13	0.017 *	21.99 ± 18.86	2.73 ± 2.54	0.001 *
FOL	nmol/L	26.18 ± 13.17	28.39 ± 13.71	0.000 *	22.64 ± 11.54	29.34 ± 11.80	0.000 *	18.95 ± 10.53	28.42 ± 13.47	0.000 *
FER	µg/L	133.06 ± 138.22	147.43 ± 168.62	0.331	158.33 ± 151.90	158.80 ± 149.74	0.796	92.89 ± 46.53	92.85 ± 46.09	0.984
Vit B12	pmol/L	498.2 ± 259.88	515.2 ± 260.12	0.155	330.9 ± 168.11	346.0 ± 195.83	0.280	375.71 ± 239.92	426.85 ± 291.65	0.009 *
CORT	µg/d	12.11 ± 4.93	12.29 ± 1.66	0.468	16.09 ± 9.98	16.32 ± 10.22	0.133	16.28 ± 8.92	16.50 ± 8.81	0.046 *
LH	IU/L	5.894 ± 3.52	5.89 ± 3.51	0.934	13.12 ± 10.70	13.43 ± 11.08	0.092	20.91 ± 11.07	20.96 ± 11.18	0.776
FSH	IU/L	11.3 ± 9.20	11.40 ± 2.91	0.122	18.15 ± 18.42	18.17 ± 18.26	0.781	18.98 ± 18.30	18.98 ± 18.30	0.286
AFP	µg/L	4.79 ± 2.24	4.74 ± 2.20	0.203	5.42 ± 3.93	5.45 ± 3.95	0.291	6.83 ± 4.88	6.82 ± 4.87	0.866
CEA	µg/L	4.7 ± 2.0	4.65 ± 1.94	0.136	6.32 ± 5.08	6.28 ± 5.14	0.532	6.37 ± 4.30	6.30 ± 4.36	0.107
CA19-9	U/mL	7.96 ± 7.14	7.90 ± 7.13	0.115	10 ± 9.22	10.12 ± 9.6	0.614	11.28 ± 11.3	11.50 ± 11.44	0.118

*** - $p < 0.05$.**

Table 2. Comparison of hemolysis interference studies and warning index setting.

Test	Hemolysis interference previously reported	Current research on hemolysis interference	Hemolysis warning index
INS	yes (-ve) [11,12,13]	yes (-ve)	1
FOL	yes (+ve) [10,11]	yes (+ve)	2
FER	yes (+ve) [11]	NS	4
Vit B12	yes (-ve) [11]	yes (+ve)	3
CORT	yes (+ve) [11]	yes (+ve)	3
FSH	NS [11]	NS	4
LH	NS [11]	NS	4
CA19-9	NS [18]	NS	4
CEA	NS [11]	NS	4
AFP	NS [11]	NS	4

-ve - negative interference, +ve - positive interference, NS - not significant.
1 - when HI = 1+, the specimen needs attention, the clinician must be informed about the deviation or the sample should be sent back.
2 - when HI = 2+, the specimen needs attention, the clinician must be informed about the deviation or the sample should be sent back.
3 - when HI = 3+, the specimen needs attention, the clinician must be informed about the deviation or the sample should be sent back.
4 - regardless of HI = 1+/2+/3+, the specimen is suitable for analysis.

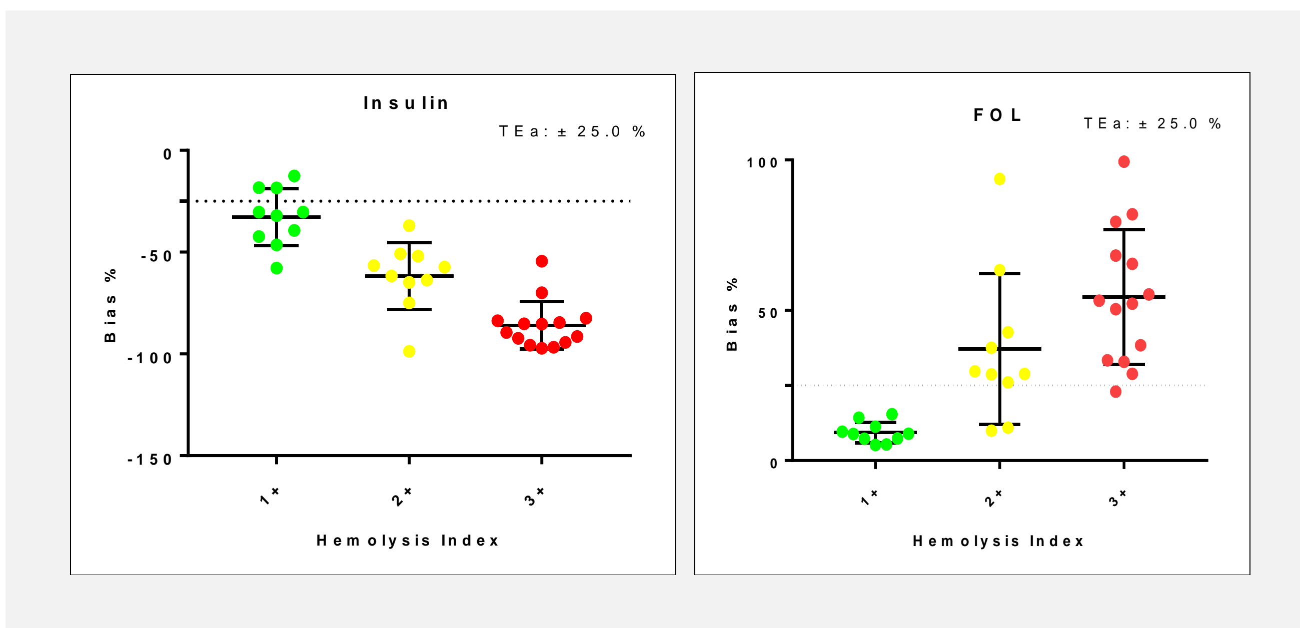

Figure 1. The effect of hemolysis on INS and FOL.

sults. Interestingly, Hasanato R. et al. used an Abbott Architect assay system and found that Vit B12 in hemolyzed specimens was reduced due to the ability of transcobalamin I/III or haptocorrin, released by red blood cell rupture, to inhibit the reaction of Vit B12 at high concentrations, resulting in negative interference with Vit B12 measurement [11]. The current study found the opposite result, where Vit B12 was higher in the hemolysis group, and the difference between the two groups was statistically significant at HI = 3+. However, in the current study, it was observed that one result, hemolysis for the HI = 2+ group, was decreased. The possible reason for this is that a Beckman DXI800 chemiluminescent assay system was used for the Vit B12 analyte and

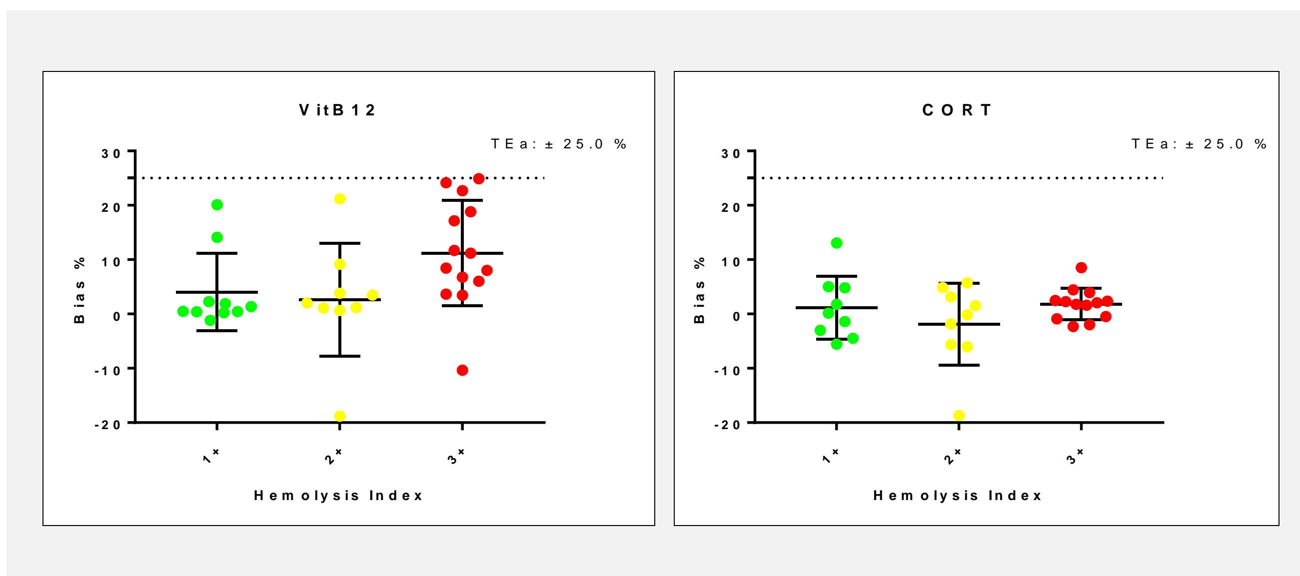

Figure 2. The effect of hemolysis on Vit B12 and CORT.

the antigen-antibody binding site is inconsistent with that of the Abbott Architect chemiluminescent assay system. In order to further investigate this phenomenon, the relevant hemolysis samples with increased Vit B12 were retrieved and inspected. Further investigation revealed that the routine blood results for these patients showed an increase in nucleated red blood cells compared to hemolysis samples that showed decreased Vit B12. It is speculated that Vit B12 contained in nucleated red blood cells in peripheral blood is released into the serum when hemolysis occurs, while hemolysis caused by mechanical damage is mostly mature red blood cells. The amount of Vit B12 contained in mature red blood cells is less than that of nucleated red blood cells, which may have led to the differing results for Vit B12.

Among the 10 analytes in our study, six were not significantly affected by hemolysis, including tumor markers FER, AFP, CA19-9, and CEA and pituitary hormones LH and FSH. The available literature suggests increased FER detection in hemolyzed specimens [11,17]. Because hemoglobin (Hb) released by red blood cell rupture is an iron-containing protein, there may be non-specific reactions between Hb and the FER antibody when chemiluminescent immunoassays are used for quantitative detection, thus resulting in positive interference [17]. In the current study, there was an increase in FER, but this increase was not statistically significant. There were also no significant differences in the accuracy of AFP, CA19-9, CEA, LH, and FSH measurements regardless of the degree of hemolysis. This is consistent with the previous literature [11], as shown in Table 2. Following clarification of the effects of the degree hemolysis on the quantitative chemiluminescent immunoassay results for 10 analytes, a hemolysis warning index was set up in combination with the HI to maximize the indicative effect of HI. For the 10 analytes in this study, the supporting reagents of the instrument were used. When assessing interference from hemolysis, an evaluation criterion of < 25% allowable total error (TEa) based on biological variation was used in conjunction with the reagent insert. For example, the reagent instructions for INS clearly state that hemolyzed specimens cannot be used, and the results of the current study also showed that the deviation in specimens affected by hemolysis HI = 1+ exceeded the TEa; thus, the warning index was set to 1. The reagent instructions for FOL also clearly state that hemolyzed specimens cannot be used. The results of the current study indicated that 80% of the specimens were affected by hemolysis HI = 2+ with a deviation greater than TEa. Therefore, the warning index was set to 2. The reagent instructions for Vit B12 and CORT reagents indicate that hemolyzed specimens should not be used. The results of the current study indicated that the effect of hemolysis was statistically significant compared with the normal group when HI = 3+, but the deviation in the affected specimen hemolysis did not exceed that of the TEa; thus, the warning index was set to 3. Similarly, the reagent instructions for FER indicate that hemolyzed specimens should not be used. The results of the current study showed no specimen affected by hemolysis deviated more than the TEa when HI = 3+; thus, the warning index was set to 4. The reagent descriptions for AFP, CA19-9, CEA, LH, and FSH say: "When used for samples with severe hemolysis (> 500 mg/dL Hb), the interference is < 10%". The current results indicated that when HI = 3+ (> 500 mg/dL Hb), no hemolysis samples with hemolysis deviated more than the TEa; thus, the warning index was set to 4.

Establishing a hemolysis warning index will provide the laboratory with a basis for specimen rejection and will facilitate clinical communication. INS is often evaluated during insulin release testing, where patients draw blood at five time points. If the sample at one time point is hemolyzed, this would affect the results. If the sample is returned for processing, usually the whole test process fails. If the sample is re-drawn, it will not only bring pain and economic burden to the patient, but will also have no clinical value over time. Therefore, the combination of the HI and warning index can allow for timely communication of the effect of hemolysis on INS with the clinician, so that the clinician can make a correct judgment on the effect on the hemolyzed sample. Clinically, many elderly patients have blood samples drawn for analysis of tumor markers such as AFP, CA19-9, and CEA, and also many infants have blood samples drawn for analysis of hormones such as LH and FSH. The difficulty of drawing blood in these populations, due to factors such as decreased vascular elasticity in the elderly and uncooperative blood drawing in young children, usually causes different degrees of hemolysis. Based on the HI combined with the hemolysis warning index, clinicians can avoid unnecessary medical disputes caused by excessive examinations.

In this study, parallel specimens of hemolysis and normal from the same patients were collected for evaluation. Compared with previous studies that have used other methods for the preparation of hemolytic specimens, such as freezing or mechanical disruption, the results of the current study truly reflect hemolytic interference. This may explain why some of the current results are inconsistent with previous reports that have evaluated interference caused by hemolysis. For example, the current hemolysis result with respect to Vit B12 detection is contrary to a report in the existing literature. It should be noted that, because it took a long time to collect samples according to this method, the number of samples obtained in this study was limited, and this may have impacted the findings.

In the evaluation of hemolytic interference, different evaluation criteria may affect the setting of hemolysis warning index and the rejection rate of hemolytic samples. It is important how to choose the appropriate standard to evaluate the interference effect, but there is no unified standard at present. In the current study, a total allowable error of 25% was chosen derived from quality evaluation requirements of NCCL and the total allowable error based on biological variation. The homepage www.westgard.com gives the following desirable specifications, according to the intra-and inter-individual biologic variation, for allowable total error in %: the chosen TEa is lower than values from the table in 5 of 10 parameters (INS: 32.9%, FOL: 39.0%, FER: 16.9%, Vit B12: 30.0%, CORT: 22.8%, FSH: 21.19%, LH: 27.92%, CA19-9: 46.03%, CEA: 24.7%, AFP: 21.9%), especially in the case of significantly affected parameters INS, FOL, Vit B12, which are considerably higher than 25%. According to a comprehensive comparison, after discussion between laboratory technicians and clinical doctors, and referring to the reagent instruction, we choose a total allowable error of 25% to ensure the clinical applicability of the evaluation results.

In summary, the effect of hemolysis on assays is complex, with interference related to the degree of hemolysis and the assay system. Laboratories should be aware of the potential for interference in all immunoassays and that experimental artefacts may cause the misinterpretation of patient results and subsequently, an incorrect diagnosis, leading to unnecessary treatments. Therefore, each laboratory should evaluate hemolysis interference according to the assay system used and should then reasonably set the hemolysis warning index applicable to the specific detection system. In addition, clinicians and laboratory technicians should closely cooperate, communicate problems in a timely manner, obtain good quality samples, and ensure correct sample transportation and storage. This would ensure the quality of the test report, reduce the error in the test results, ensure the reliability of the test results, and provide a reliable basis for the diagnosis and treatment of patients.

Acknowledgment:

This work was supported by the National Natural Science Foundation of China (Grant No. 61972187).

Declaration of Interest:

Authors declared no conflict of interests.

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