

Effect of Hemolysis on Plasma Cardiac Troponin Levels at Clinically Relevant Concentrations: An Experimental Study

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Abstract

Hemolysis is one of the most commonly encountered pre-analytical sources of interference in the Clinical Biochemistry laboratory. Hemolysis is known to cause a variable interference in both cardiac troponin I (cTnI) and troponin T (cTnT) assays. Each laboratory should determine and publicize these effects. In this experimental study we determined the effect of in-vitro sample hemolysis on both types of cardiac troponin assays and whether the effect was clinically significant.

Keywords:

Hemolysis; Cardiac troponin; cTnT; cTnI; Interference; Oman

Introduction

In-vitro hemolysis is one of the most common types of pre-analytical sources of error encountered in the clinical laboratory [1]. We frequently receive hemolyzed blood samples at the Clinical Biochemistry Laboratory, from the Emergency Department at Sultan Qaboos University Hospital (SQUH). In many of these occasions, different biochemical parameters including cardiac troponin levels are requested. Cardiac troponin levels, whether troponin I (cTnI) or troponin T (cTnT), is considered to be the most sensitive and specific biochemical marker that is currently used for diagnosis, management and stratification of patients with acute coronary syndrome (ACS) [2]. According to the most recently published 2012 task force report by the American College of Cardiology, changes of >20% in cardiac troponin levels, are considered to be statistically significant and suggestive of an ischemic event including post-percutaneous coronary intervention [2].

Hemolysis is known to cause interference in the measurement of both cTnI and cTnT assays. Few published studies have shown that current troponin assays have different susceptibilities to interference by hemolysis. Some studies showed negative interference with cTnT high sensitive (cTnT-hs) assays (up to 50%) while other studies showed positive interference with cTnI assays [3,4]. Due to such diverse responses to hemolysis as an interferent, every laboratory should quantify and document the effect of hemolysis on its cardiac troponin assay(s), as part of good and valid laboratory practices.

Objective

To evaluate the effect of varying degrees of hemolysis on second-generation cTnI and on cTnT-hs assays at all clinically relevant troponin concentrations using two different automated platforms, namely Access (Beckman Coulter) and Cobas e601 (Roche) analyzers that are available at SQUH and the Royal hospital respectively, the two tertiary care centers, in Muscat, Oman.

Methodology

This study was a non-funded study approved by the ethics committee of the College of Medicine and Health Sciences at Sultan Qaboos University (SQU). The clinically relevant cTnI and cTnT

concentrations at which there was a need to investigate the effect of hemolysis were assessed. This was determined according to the diagnostic cut-off levels stated in several international guidelines, namely the 99th percentile and the acute myocardial infarction (AMI) cut-offs [2]. In addition, we investigated the effect of hemolysis at much higher levels of troponins, which are the levels that are relevant for the diagnosis of post procedural MI, namely post percutaneous coronary intervention (PCI) and post coronary artery bypass graft (CABG) [2]. The 99th percentile manufacturer-defined cutoffs are 40 and 14 ng/L for cTnI and cTnT respectively. The AMI cutoffs are 500 and 100 ng/L for cTnI and cTnT respectively [5,6].

Pools of un-hemolysed heparinized plasma samples containing the relevant troponin concentrations were collected after their initial analysis for cTnI and cTnT, and stored immediately at 2°C. The pools were originally selected from patients' blood samples that were referred to the laboratories of the two hospitals, SQUH and Royal Hospital for measurement of plasma troponin levels. In these laboratories, as per practice protocol, samples are usually stored for two weeks before being discarded. These pools were prepared on the day the samples were scheduled for discard.

Four major pools were prepared and tested for both types of cardiac troponin assays: 99th percentile pool 30 4050 ng/L for cTnI; 14 ng/L for cTnT), AMI pools (500 ng/L for cTnI; 108 ng/L for cTnT) and higher troponin concentration pools for post PCI and post Coronary artery bypass graft (CABG) MI (4600, 7300 ng/L for cTnI; 1208, 2309 ng/L for cTnT).

Prior to testing the effect of hemolysis as an interferent, confounders

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such as sample stability and analytical coefficient of variation (CV) at each relevant cut off level were accounted for. This was achieved by carrying out both stability and precision studies. The manufacturers' package inserts for both cTnI and cTnT assays recommend analyses of plasma samples within 2 hours if kept at room temperature, due to possible evaporation effects [5,6]. Accordingly, we immediately refrigerated all our samples after initial analyses. We mainly determined troponin stability at two weeks from sample collection, when non-hemolysed heparinized plasma samples are stored at 2-7°C. This was important to determine since we were measuring the effect of hemolysis on troponin levels in refrigerated samples scheduled for discard after 2 weeks from collection. This was achieved by testing samples from each pool containing troponin values around the relevant clinical cut-offs in duplicates upon their initial analysis in the laboratory and after 2 weeks from storage at 2°C, and then comparing between the mean values using the paired t-test.

According to the published international guidelines, the maximum total analytical imprecision (%CV) at the 99th percentile cut off, should be less than 10% [2]. The %CV at the 99th percentile cutoff, was estimated by measuring troponin levels in heparinized plasma samples at the 99th percentile twenty times. The mean, standard deviation (SD) and %CV were then estimated using Microsoft office Excel statistical package. The % CV was also estimated for each of the AMI cutoff pools.

Pools of un-hemolysed plasma samples containing troponin concentrations around the 99th percentile cut off values of 30, 40 and 50 for cTnI and 14 ng/L for cTnT, were collected and stored at 2°C, immediately after analysis. The same procedure was followed for the AMI cutoff pools, which were 500 ng/L and 108 ng/L for cTnI and cTnT respectively.

To examine the effect of hemolysis, hemolysates were prepared from EDTA treated whole blood samples. Packed red blood cells (PRBC) were washed three times with 0.9% normal saline and hemolysing reagent was added gradually to create different hemolysates containing increasing hemoglobin (Hb) concentrations ranging from low to high (0.2, 0.6, 1.0, 1.4, and 2.0 g/dL). The hemoglobin concentration in each hemolysate was measured using Radiometer ABL 800 blood gas analyzer. The final desired hemoglobin concentrations to be achieved in the plasma sample were determined from previously published studies. The hemolysing reagent contained the following components: 10 mmol/L sodium phosphate monobasic, 40 mmol/L sodium phosphate dibasic and 0.4% (w/v) nonylphenol polyethylene glycol ether.

At the time of the study, the hemolysis index of each sample was not measured routinely on the automated machine. To render our results comparable to other published studies, we therefore, calculated the desired Hb concentration in each hemolysate from the following equation:

Hb. Conc. desired = hemolysis index desired (as per previous publications) X 19 g/dl (1.9 g/L)/150 (AU)

A hemolysis index of 150 arbitrary units (AU), in one published study, was equivalent to a hemoglobin concentration of 19.0 g/dl (1 g/L), within the sample [4].

Ten µl from each prepared hemolysate was added to 90 µl of plasma sample from each pool in duplicates. Since this is considered a 1:10 dilution, each prepared hemolysate pool was deliberately prepared to be 10 times the final desired hemoglobin concentration in plasma. The final hemoglobin concentration in plasma was then assumed to be as follows ranging from low to high: (0.2, 0.6, 1.0, 1.4 and 2.0 g/dl). Both

cTnI and cTnT concentrations were determined for each duplicate pre and post addition of the hemolysate using Access (Beckman Coulter, USA) and Cobas e601 (Roche, Germany) analyzers for cTnI and cTnT respectively.

Statistical Analysis

The results were analyzed using Excel and statistical software SPSS program. All results were also adjusted for any dilutional effect on troponin concentrations secondary to the addition of hemolysate regardless of hemoglobin concentration. For each type of troponin assay, analysis was carried out overnight, in duplicates, in one batch to minimize inter-assay variability and all internal and external quality control procedures were within acceptable limits.

Results

Both cTnT and cTnI levels in all pools were stable when samples were stored at 2-7°C for up to 2 weeks. The differences between the mean values of each pool were tested using paired t-test (Tables 1 and 2). The mean values and standard deviation (SD) for each pool are also reported in Tables 1 and 2. The t and 2-tailed p values were not significant. The mean and SD values also did not change dramatically which meant that the 2 week old pools could be used for the purpose of this experiment.

The precision study revealed that the %CV of both troponin assays at the 99th percentile cut off (40 ng/L for cTnI and 14 ng/L for cTnT) was 9.7% and 5.2% respectively. For the other higher troponin I level pools the troponin I values and the corresponding (%CV) were as follows: 500 ng/L (2.8%), 4600 ng/L (1.4%) and 7300 ng/L (2.5%). For the higher cTnT pools, the values and the corresponding (%CV) were as follows: 108 ng/L (2.7%), 1208 ng/L (2.8%) and 2309 ng/L (1.7%). Hemolysis generally caused a concentration dependent-negative interference with both cTnI and cTnT assays at all levels, the effect was more pronounced with cTnT; Figures 1 and 2. For cTnI, the effect was more obvious at the AMI cutoff (500 ng/L) and at the higher troponin concentration pool (7300 ng/L). The effect became significant at higher troponin concentrations, causing ≥ 20% decrease in troponin levels, when hemoglobin concentration in the sample was 2.0 g/dL (20 g/L), which is equivalent to a hemolysis index of around 1500. Interestingly, hemolysis did not have a significant effect on TnI at the 99th percentile pool (40 ng/L) except at a single point (0.6 g/dL hemoglobin concentration), and had a less than 10% negative interference effect on the 4600 ng/L pool. However, it is worth noting that despite the overall less than 10% effect on the 4600 ng/L pool, the effect was generally a clear trend of increasing negative interference with increasing hemoglobin concentrations (Figure 1). The effect on cTnT assay was more obvious around the AMI cut off (108 ng/L) and around the 99th percentile cutoff (14 ng/L). The negative effect was more obvious with increasing degrees of hemolysis with a maximal effect at around 1.4 g/dL (hemolysis index 1050) then it tends to decrease higher hemoglobin concentration (2 g/dL).

Discussion

The effect of hemolysis on cardiac troponin levels is not a newly described phenomenon. There are some previously published studies that have addressed this clinically relevant problem [3,4,7]. However, it is essential that every laboratory is aware of the effect of hemolysis on its troponin assays and to study this effect independently. Hemolysis has been reported to interfere positively or negatively, significantly or minimally with troponin assays, depending on various factors. These factors include, but are not limited to the type of assay, type of automated platform, effect of free hemoglobin versus hemolysate, and

Pools in order (ng/L)	Average TnI (Fresh) (SD) ng/L	Average TnI (2weeks) (SD) ng/L	T value (critical t, Df =1, 95 % confidence= 12.7)	2 tailed P value
1(40)	45 (7.0)	45 (7)	0	0
2 (500)	475 (7.0)	445 (3.5)	4.3	0.14
3 (4600)	4636 (50)	4529 (41)	0.8	0.60
4 (7300)	7345(63)	7300(14.1)	1.3	0.40

Table 1: Troponin I (Tn I) stability study.

Pools in order (ng/L)	Average TnT (Fresh) (SD) ng/L	Average TnT (2 weeks) (SD) ng/L	T value (critical t, Df=1, 95 % confidence=12.7)	2 tailed P value
1(14)	13.5 (0.70)	12.90 (0.21)	1.90	0.32
2 (108)	108 (0.70)	106 (2.30)	1.80	0.33
3 (1208)	1212 (5.0)	1149 (4.2)	9.6	0.07
4 (2309)	2317 (11.3)	2285 (19.8)	1.50	0.40

Table 2: Troponin T (TnT) stability study.

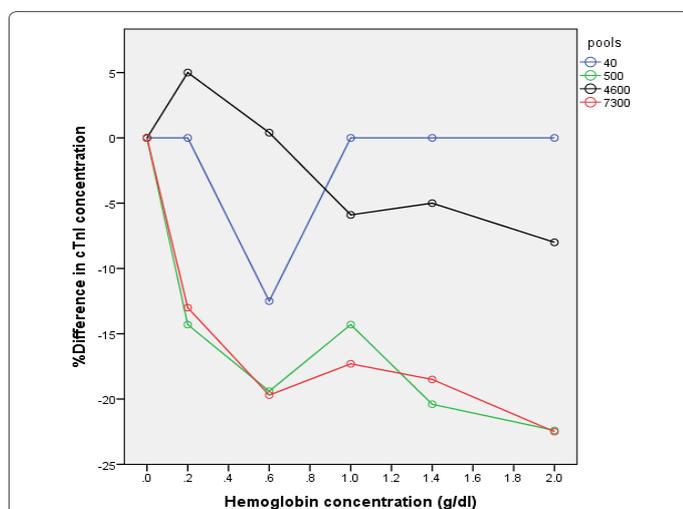


Figure 1: Interferogram showing the magnitude of effect of added hemolysate on cTnI concentration.
 $\% \text{ difference} = \frac{\text{cTnI concentration (without hemolysate)} - \text{cTnI (with hemolysate)}}{\text{cTnI (without hemolysate)}} \times 100$

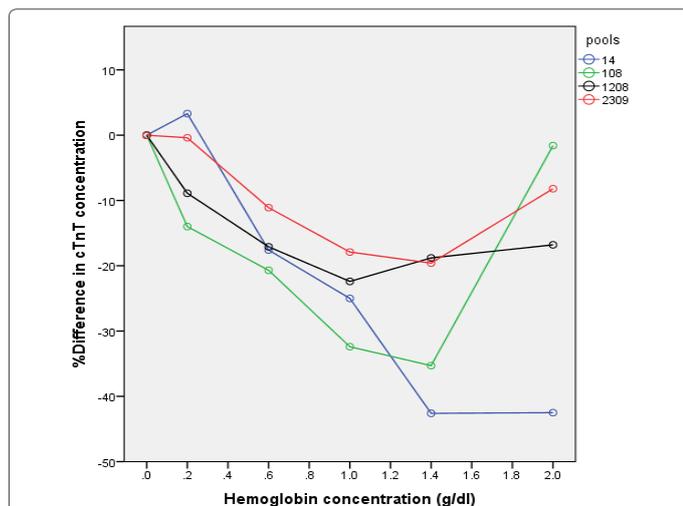


Figure 2: Interferogram showing the magnitude of effect of added hemolysate on cTnT concentration.
 $\% \text{ difference} = \frac{\text{cTnT concentration (without hemolysate)} - \text{cTnT (with hemolysate)}}{\text{cTnT (without hemolysate)}} \times 100$

degree of hemolysis [7-12].

In our study, we showed that generally there was an increase

in the degree of negative interference with increasing hemoglobin concentrations for both types of cardiac troponins using the two different automated platforms, at mostly, all troponin concentrations. However, the negative interference effect of hemolysis on cTnT was more obvious than on cTnI and appeared in all the pools at the different concentrations. The effect continued to be visible even after subtracting the dilutional effect of the addition of hemolysate to the original pool. Interestingly, at the lowest troponin T concentration pools initially there was a very small rise of less than 10 % in troponin concentrations before the actual negative interference is apparent at higher hemoglobin concentrations. This can be attributed to the analytical imprecision at those low levels rather than the effect of hemolysis per se. Because of the inherent analytical imprecision at these lower analyte levels, there is very little room for allowing other analytical errors without exceeding the total allowable error for the method. This, along with the 99th percentile diagnostic criteria for AMI, makes the determination of various interferences, including hemolysis, at the lower levels essential [8]. Our findings are mostly consistent with other previously published studies that showed that cTnI assays seemed to be more resilient to hemolysis than cTnT assays and sometimes the interference with cTnI assay would be a positive rather than a negative interference [4,7-9].

The actual mechanism of hemolysis induced negative interference with troponin assays remains largely unknown and not well understood. That being said, Sodi et al. [10] explained experimentally the mechanism of hemoglobin and hemolysis induced negative interference in cTnT assay. This was demonstrated to be due to the release of proteases from the red blood cells that then degrade specific antigenic regions in the cTnT molecules preventing them from being detected by the assay. In addition, hemoglobin usually causes an added negative interference distinct from the effect of hemolysis. Also, it is thought that free hemoglobin can “quench” the light produced in luminescent assays thereby reducing the intensity of the final signal and hence causing a negative bias [11].

Another study showed the susceptibility of cTnI to proteolytic degradation [12]. Indeed, although the access Accu TnI package insert claims that a hemoglobin concentration of up to 500 mg/dl did not affect the results of cTnI assayed, however, the information package clearly states that cTnI is “highly susceptible to proteolysis and enzymatic degradation” [5]. Although the manufacturer claims to have monoclonal antibodies directed against the more stable region of the molecule, this does not eliminate completely the effect proteolysis on cTnI, it will only diminish it, as demonstrated by our and other previously published studies. Indeed, proteolysis will produce various cardiac troponin I molecules with varying degrees of stability hence it is not unreasonable to assume that unpredictable interactions between these molecules and the antibodies in the assay can occur

[12]. Furthermore, manufacturers usually add human methemoglobin to test the effect of "hemoglobin" as an interferent. However, this will not mimic the commonly seen in-vitro or the less common in-vivo type of hemolysis usually seen in clinical practice. This is because red blood cells contain constituents other than just hemoglobin alone. This would explain the apparent contradiction between what is mentioned in the package insert and the results of our experiment. This could also explain, partially at least, the variable response in the cardiac troponin I pools. For instance we see that, pools 40 and 4600 ng/L were the least affected by hemolysis. Whereas, we see that with the other two pools the negative interference is apparent. There was one dip in pool 40 at 0.6 mg/dl, which could be a random error or a real result. It seems that troponin I is susceptible to hemolysis but in a less predictable manner than troponin T. This could be due to various reasons including the structure of troponin I versus cardiac troponin T, the antibodies used in each assay, and the individual susceptibility of each molecule to hemolysis itself.

In our study, we added hemolysate to non-hemolysed plasma pools and these hemolysates contain both hemoglobin and proteolytic enzymes, hence both mechanisms of interference are plausible. Also, one point which is worthy of note, was that with the initial sharp negative bias in troponin concentrations with hemolysis, after a certain point, further increases in total hemoglobin concentrations resulted only in minimal or no additional decline in troponin concentrations. This is especially clear with the cTnT assay where beyond a hemoglobin concentration of 1.4 g/dl (14.0 g/L, which is equivalent to a hemolysis index of around 1050 Arbitrary Units), the negative effect of hemolysis on troponin level seems to have plateaued in all troponin pools. This was also observed but to a lesser extent with the cTnI assay. There are some plausible explanations for this. It is thought that red blood cell corpuscular components can bind with low affinity to unoccupied antibody sites causing a positive interference in luminescent immunoassays [8]. Although this effect is usually seen at low analyte levels, since the analyte binds with high affinity to the specific antibody, however it is possible that when hemoglobin concentrations are high enough this effect might become sufficiently important to cause a significant positive interference.

Previous studies did not show this apparent biphasic pattern of hemolysis-induced interference with cTnT assay. Most studies showed a concentration-dependent decrease in troponin levels with hemolysis [3,4,7-9]. The positive interference was mainly seen with the cTnI assay [4]. This could be because in previous studies the maximum tested hemoglobin concentration as an interferent did not go beyond 1.4 g/dL (14 g/L). In this study we studied hemolysis as an interferent up to a maximum hemoglobin concentration of 2.0 g/dL (20 g/L) which is much higher than in previously published studies. Since at these high hemoglobin levels more hemolysate was added, the possibility of non-hemoglobin red blood cell components interacting with unoccupied antibody antigenic sites and causing false positive reactions is not an unreasonable explanation.

Also, although we have no record of the frequency of hemolysed samples received at our laboratory, it is known from various studies that sample hemolysis is the most frequent cause of sample rejection and is a common pre-analytical source of error [1,13,14]. Cardiac troponin levels are used for critical decision making in the diagnosis and management of patients with ACS. Serial cardiac troponin measurements are used frequently in clinical practice and it is the pattern of rise and fall in their levels that is used for diagnosis and management of myocardial damage. Therefore, the potential for

confusing troponin results in the presence of hemolysis especially for basal samples has to be considered. The solution to this problem, however, is not straightforward. As shown in our study and previously published studies, the hemolysis-induced negative interference with troponin assays seems to be significant only when hemoglobin concentration is beyond 0.3 g/dL. This is the level beyond which further hemolysis can be distinguished visibly by the human eye [15]. One might argue that beyond this level samples should be rejected anyway because they would be considered moderately hemolysed by the naked eye or by photometric techniques (hemolysis index). However, this is not always practical; as hemolysis is frequently encountered in practice and rejecting every hemolysed sample seems unreasonable. A more reasonable solution would be to analyse the mildly hemolysed samples and releasing troponin results with a commentary note. Only grossly hemolysed samples should be rejected and another non-hemolysed sample should be recollected for more accurate results. Meanwhile, comments could be reported on the status of hemolysis, for example: 'hemolysis can negatively interfere with troponin result', 'advise close follow up of serial troponin values', 'sample received grossly hemolysed', 'please send another non hemolysed sample as this degree of hemolysis can significantly affect troponin results'.

The main limitations of our study include the small number of pools tested due to logistical and practical reasons. The use of hemolysate addition as a way of investigating the effect of hemolysis is in our view at least, much better than just adding methemoglobin as an interferent as most vendors do [15]. However, this does not completely mimic the effect of in-vitro or in-vivo hemolysis as the mechanism of destroying the red blood cells is not identical. Some might argue that we only tested sample stability at refrigerated temperatures and not at room or frozen temperatures. However, this was done to mimic everyday laboratory practice. Also, we wanted to follow the manufacturer directions so that any deviations in results could not be attributed to lack of adherence to standard recommendations.

Conclusion

Blood samples for plasma troponin analysis should be separated and analysed for troponin within two hours of blood collection. Mildly hemolysed samples should be analysed and results released with a commentary note. Moderately or severely hemolysed samples should be rejected as the interference in these samples is clinically significant. Each laboratory has to be aware of the effect of hemolysis on its individual troponin assay.

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