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Comparing the effect of delayed serum separation on Creatinine measurement by Jaffe and Sarcosine oxidase enzymatic methods with different time and temperature

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***Abstract*—**Creatinine is a nitrogenous organic waste product and increased in serum under abnormal renal function. Commonly used methods for creatinine measurement are Jaffe and Sarcosine Oxidase enzymatic methods. Delays in serum separation may cause changes in serum creatinine level. This could lead to misclassification of the stages of Chronic Kidney Disease. The study was carried out in normal pooled serum to find the changes in the serum creatinine levels of normal individuals, if the blood is stored for different periods and the serum separation is delayed. Ten milliliters (10 mL) of blood from 3 healthy individuals were pooled, equally divided and were stored. Creatinine concentrations in the serum immediately separated from the blood samples which were stored for 2 and 6 hours & 1, 2 & 3 days, at room temperature ($\approx 27 - 29^{\circ}$C) & in a refrigerator were measured. The results indicated that the Sarcosine Oxidase enzymatic method gives more accurate values of serum creatinine level than the Jaffe method. Samples can be stored at room temperature for 6 h without significant change in creatinine concentration ($p<0.5$) and refrigerator is the best method for delayed serum separation ($p<0.5$).

***Keywords*—**Creatinine, Jaffe method, Sarcosine Oxidase, Enzymatic assay, Chronic Kidney Disease

I. Introduction

Creatinine is a nitrogenous organic waste product of the body that is produced by the breaks down phosphocreatine. Creatine is synthesized by the liver, kidney and pancreas (Charles *et al*., 2015). Most routine assays for creatinine employ serum samples. Generally, the laboratory receives the specimen in the form of whole blood and then the serum is separated. For clinically useful and reliable test results, the interval between blood collection and serum separation must be controlled. Ideally the serum should be separated as soon as possible after the clot formation (usually 30 minutes) to avoid clot induced changes in the concentration of serum analytes (Laura *et al.*, 2015). In practice, manually very few routine specimens can be processed within this time interval manually. An interval as long as 2hours between blood collection and serum separation is still considered acceptable, because storing serum in contact with the clot for 2hours at room temperature(25°C) generally results in clinically insignificant changes in most commonly measured constituents in serum. However, there can be a considerable delay in sample delivery, either because of improper handling procedures or human errors. Such extreme cases, where contact of serum with clot may exceed 24 hours, can result in clinically significant alterations of serum analytes (Nadja *et al*., 1988).

Several chemical methods are available for the estimation of creatinine in serum and urine, which are based on the Jaffe's reactions (Bartel *et al*., 1972), Sarcosine Oxidase an enzyme method (Marakala *et al*., 2012), High Pressure Liquid Chromatography (HPLC) and Isotope Dilution Mass Spectrometry (IDMS). Although HPLC and IDMS are the most accurate methods (Myers *et al*., 2006), these techniques are not suitable for routine clinical laboratory because they are expensive and require complex sample preparation and specialized personnel. Most common are Jaffe's and Sarcosine Oxidase enzymatic methods. Jaffe method is more susceptible to many interferences. More than 50 chromogenic interfering substances have been documented. Presence of high concentration of haemoglobin, bilirubin, lipid, ketone derivatives, glucose, pyruvic acid, ascorbic acid and proteins interfere with the creatinine concentration in vivo and in vitro (Bartel *et al*., 1972). Certain drugs such as Streptomycin (an aminoglycoside), Cefazolin, Cefamandole, Cephalothin and Cefoxitin have been shown to interference with creatinine determination at various concentrations. Most of the above are cause positive interference except bilirubin. The presence of positive interfering substances in serum can lead to the over estimation by as much as 15-25 % by various Jaffe's methodological applications (Myers *et al*., 2006). In practice, very few routine specimens can be processed within this time interval manually. There can be a considerable delay

in sample delivery, either because of improper handling procedures or human errors. Such extreme cases, where contact of serum with clot may exceed 24 hours, can result in clinically significant alterations of serum analytes (Shephered *et al*., 2007).

Historically, several studies have been done regarding the delay in separating blood samples affects creatinine measurement, but no any complete comparative studies have been carried out to compare the effect of storage time and temperature on serum creatinine by both Jaffe & Sarcosine Oxidase enzymatic methods in delayed serum separation. This study will help to give an insight on the effects of delayed serum separation on creatinine measurement by Jaffe (Bartel *et al*., 1972) and Sarcosine Oxidase enzymatic (Marakala *et al*., 2012) methods and to find appropriate method, when delays occur during serum separation. Further, this study will help to determine the maximum possible storage time to store the blood sample as whole blood in serum creatinine measurement before the analysis at refrigerator ($2 - 8°$C) and at room temperature ($\approx\ 27 - 29°$C). Delays in serum separation can cause significant increases in measured creatinine by the commonly used Jaffe method. This could lead to misclassification of CKD stage and possibly further unnecessary investigations. The use of the enzymatic method appears to provide more reliable CKD classification in situations where specimens have taken time to reach the laboratory (Shephered *et al*., 2007). This study will help to give an insight on the effects of delayed serum separation on creatinine measurement by Jaffe (Bartel *et al*., 1972) and enzymatic (Sarcosine Oxidase) methods (Marakala *et al*., 2012) and to find an appropriate method, when delays occur during serum separation. This study will help to determine the maximum possible storage time to store the blood sample as whole blood in serum creatinine measurement before the analysis at refrigerator and at room temperature.

Objectives of the study

Objective of this study is to comparing the effect of delayed serum separation on creatinine measurement by Jaffe method with an enzymatic method. To aim this general objective further divided into three specific objectives. Those are 1) To assess the changes in serum creatinine level at baseline level (0 hour) and blood stored for 2 & 6 hours and 1, 2 & 3 days at room temperature ($\approx\ 27 - 29°C$) and refrigerator ($2 - 8°$C) by using Jaffe method and Sarcosine Oxidase enzymatic method 2) To find the suitable storage time and temperature to preserve the blood samples for serum creatinine measurement 3) To find the suitable estimation method for serum creatinine measurement when the serum is contact with cells for longer period. So, this study was to design according to the specific objectives.

II. MATERIAL AND METHOD

Study set up and samples

This is a laboratory- based experimental study. The study was carried out from August 2018 to July 2019. Data collection was carried out in June 2019. The blood samples were collected from 3 voluntary healthy male and female students from the Faculty of Medicine, University of Jaffna.

A. BLOOD SAMPLING AND ANALYSIS

This study was carried out with pooled normal serum. Hence, sample size calculation was unnecessary but the determination of sample volume was needed. So that sample volume was calculated according to the test procedure. A hundred microlitre (100 μL) of serum sample was needed for each creatinine test by Jaffe method, so for all the 24 tests (including duplication) by Jaffe method 2.4 mL of serum was needed. Sixty microlitre (60 μL) of serum sample was needed for each creatinine test by Sarcosine Oxidase enzymatic method, so for all the 24 tests (including duplication) around 1.5 mL of serum was needed. Totally 4 mL of serum was needed. Therefore, approximately 10 mL of blood was needed to separate the serum. Thus 3 healthy voluntary individuals were included in this study. Ten millilitre (10 mL) of blood was collected from each person. This was a voluntary based study. An open invitation was given to students of the Faculty of Medicine, University of Jaffna. The questionnaire was provided and Informed written consent was obtained from each individual who was accepted. Ethical clearance was obtained from Ethical Review Committee, Faculty of Medicine, University of Jaffna. Blood was collected from the individuals and pooled serum was prepared at 30 minutes, 2 & 6 hours and 1, 2 & 3 days. The blood samples were kept at room temperature for 30 minutes. Then clotted samples were centrifuged at 3000 rpm for 10 minutes and serum was separated (Gunatilake *et al*., 2005). Two set of tubes were kept at room temperature ($\approx 27 - 29°C$) and refrigerator ($2 - 8°$C) respectively. After 2 & 6 hours and 1, 2 and 3 days of storage, clotted samples were centrifuged at 3000 rpm for 10 minutes and serum was separated. Pooled serum was prepared by mixing required amount of serum samples on each time interval for both tubes at room temperature ($\approx\ 27 - 29°C$) and ($2 - 8°$C) refrigerator. Then the creatinine concentration was measured by using Jaffe method and enzymatic method. Pooled serum was prepared by mixing all serum samples at each time interval.

B. STATISTICAL ANALYSIS

The data and results obtained were analyzed using descriptive statistics such as tables, graphs, mean comparison (One way ANOVA and Tukey's honestly significant difference (HSD) post hoc test), standard deviation. Data was entered in Statistical Package for Social Sciences (SPSS) version 23. The p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

III. RESULTS

Serum samples collected as soon as the collection of the blood were analysed for the baseline creatinine concentration

and considered as 0 hour. The mean creatinine was 1.213 (±0.004) mg/dL by Jaffe method and 1.065 (±0.007) mg/dL by Sarcosine Oxidase enzymatic method (Table 1). The reference range of serum total creatinine in adult individual is 0.9-1.3 mg/dL (80-115 μmol/L) (Burtis *et al*., 2008). The concentration of creatinine in the serum sample collected from the pooled blood at 0 hour was within the normal range and confirmed that the blood samples collected were from normal individuals.

Creatinine concentration in the Serum prepared from the blood samples stored at room temperature

Total creatinine concentration of the serum samples prepared from the blood samples stored at room temperature were measured at specified time intervals. The mean serum creatinine concentrations (mg/dL) measured by Jaffe method prepared from the blood samples separated at 2 & 6 hours, 1, 2 & 3 days were 1.227 (±0.004) & 1.472 (±0.005), 1.735 (±0.014), 2.12 (±0.064) & 2.24 (±0.069) mg/dL respectively. There has been increase in the serum creatinine level when the blood samples were stored for 6 hours was observed (Table 1). The increase in serum creatinine levels became significant in the samples from those stored for 6 hours (p<0.05).

When the creatinine concentrations of the serum samples prepared from blood specimens stored for 2 & 6 hours, 1, 2 & 3 days were 1.108 (±0.006) & 1.217 (±0.004), 1.310 (±0.036), 1.410 (±0.028) & 1.473 (±0.005) mg/dL respectively measured by Sarcosine Oxidase enzymatic method (Table 1). Statistically significant increase in serum creatinine level was observed in the blood samples from which were stored for 6 hours or beyond this time (Table 1, Figure 1, $p < 0.05$).

Creatinine concentration in Serum samples prepared from the blood samples stored by refrigiration

Total creatinine concentration of the serum samples prepared from the blood samples stored in the refrigerator were measured at specified time intervals. The mean serum creatinine concentrations (mg/dL) measured by Jaffe method prepared from the blood samples separated at 2 & 6 hours, 1, 2 & 3 days were 1.246 (±0.014) & 1.324 (±0.006), 1.487, 1.670 & 1.720 mg/dL respectively. There has been increase in the serum creatinine level when the blood samples were stored for 1 day was observed (Table 2). The increase in serum creatinine levels became significant in the samples from those stored for 1 day (p<0.05).

When the creatinine concentrations of the serum samples prepared from blood specimens stored for 2 & 6 hours, 1, 2 3 days were 1.092 (0.009) & 1.100 (±0.007), 1.187 (±0.010), 1.230 (±0.042) & 1.324 (±0.007) mg/dL respectively measured by Sarcosine Oxidase enzymatic method (Table 2). Statistically significant increase in serum creatinine level was observed in the blood samples from which were stored for 2 days or beyond this time (Table 2, Figure 2, $p < 0.05$).

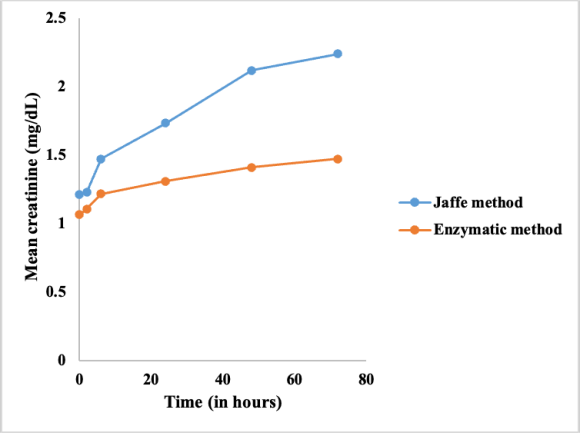

Figure 1: Changes in mean creatinine concentration at room temperature

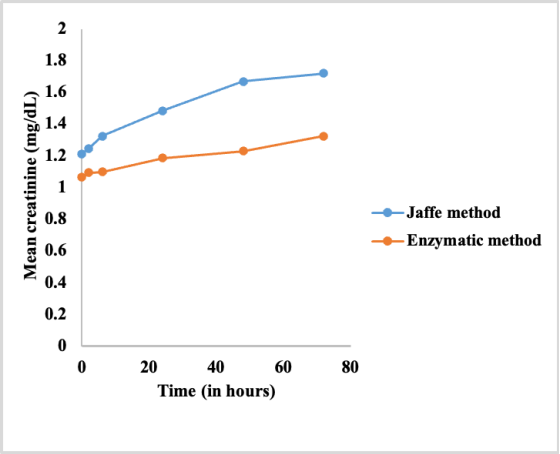

Figure 2: Changes in mean creatinine concentration at $2 - 8^{\circ}$C

IV. DISCUSSION

The concentration of serum creatinine was increased when the clot was in contact with cells for longer period. The results of the present study clearly demonstrate the effect of delayed serum separation on creatinine measurement by the Jaffe's method with the Sarcosine Oxidase method. When unseparated blood specimens were stored at room temperature, significant alteration of creatinine concentration was obtained beyond 6 hours by Jaffe's and Sarcosine Oxidase method. But the changes in creatinine concentration in percentage are less significant when the creatinine is measured by Sarcosine Oxidase method than the values those observed with the Jaffe's method. The same findings were presented by Sphered *et al*., (2007), blood samples were collected and stored. Then separated at defined time points after collection (15 min, 4h, 8h, 14h, 24h and 31h). Creatinine concentration was measured by Jaffe and enzymatic method. A 24h delay in centrifugation of whole blood resulted in significant increases in measured creatinine when assayed by Jaffe (p<0.025). The enzymatic method showed no significant difference (p>0.2) in creatinine concentration. Over a period of 31 hours creatinine concentration was stable using enzymatic

Table I: Creatinine concentration of serum prepared from the blood samples stored at room temperature by Jaffe's method and Sarcosine Oxidase enzymatic methods

Time	Jaffe's method Creatinine concentration (±SD) (mg/dL)	P# value	Change in Creatinine (%)	Enzymatic method Creatinine concentration (±SD) (mg/dL)	P# value	Change in Creatinine (%)
0	1.213 (±0.004)	-	-	1.065 (±0.004)	-	-
2	1.227 (±0.004)	0.990	1.14%	1.108 (±0.006)	0.040	3.89%
6	1.472 (±0.005)	0.010	17.53%	1.217 (±0.004)	0.150	12.48%
1 day	1.735 (±0.014)	0.000	30.08%	1.310 (±0.036)	0.000	18.70%
2 days	2.120 (±0.064)	0.000	42.78%	1.410 (±0.028)	0.000	24.46%
3 days	2.240 (±0.069)	0.000	45.84%	1.473 (±0.005)	0.000	27.69%

Table II: Creatinine concentration of serum prepared from the blood samples stored in the refrigerator by Jaffe's method and Sarcosine Oxidase enzymatic methods

Time	Jaffe's method Creatinine concentration (±SD) (mg/dL)	P# value	Change in Creatinine (%)	Enzymatic method Creatinine concentration (±SD) (mg/dL)	P# value	Change in Creatinine (%)
0	1.213 (±0.004)	-	-	1.065 (±0.004)	-	-
2	1.246 (±0.014)	0.960	2.65%	1.092 (±0.009)	0.960	2.47%
6	1.324 (±0.006)	0.270	8.38%	1.100 (±0.007)	0.900	3.18%
1 day	1.487 (±0.007)	0.010	18.43%	1.187 (±0.010)	0.070	10.27%
2 days	1.670 (±0.085)	0.000	27.36%	1.230 (±0.042)	0.020	13.41%
3 days	1.720 (±0.068)	0.000	29.48%	1.324 (±0.007)	0.000	19.56%

creatinine assays. A significant increase was seen by 24 hours using Jaffe method (p< 0.025).

The current study showed, there were statistically significant change (p< 0.05) in creatinine concentration in serum sample stored at room temperature ($\approx 27-29^{\circ}C$) after 6 hours by Jaffe and after 2 days at refrigerator ($2-8^{\circ}$C). The changes in increased creatinine level in percentage was range from 2.65% to 29.48% by Jaffe method and 2.47% to 19.56% by enzymatic method at refrigerator ($2-8^{\circ}$C). Whenever delays occur it's better to keep the blood samples in refrigerator ($2-8^{\circ}$C). This was in accordance with study done by Ford and Berg, (2008), used clotted samples were left at room temperature up to 48 hours (4, 8, 16, 24, 36 and 48 hours) prior to centrifugation. Serum creatinine was measured by Jaffe method. A significant increase in creatinine concentration (p < 0.001) occurred beyond 16 hours of delay in sample separation. The mean increase in creatinine level by 24 hours was 11% and by 48 hours was 29%.

The relative increase in the creatinine concentration at room temperature from 0 to 3 days ranged between 1.14% to 45.84% and 3.89% to 27.69% (2h to 3 days) by Jaffe's and Sarcosine Oxidase methods respectively. The changes in creatinine concentration measured by an enzymatic method are more reliable than the Jaffe method. These finding were in line with Sphered *et al.*, (2007), blood samples were collected and stored. Then separated at defined time points after collection (15 min, 4h, 8h, 14h, 24h and 31h). Creatinine concentration was measured by Jaffe and enzymatic method. A 24h delay in centrifugation of whole blood resulted in significant increases in measured creatinine when assayed by Jaffe (p<0.025). The enzymatic method showed no significant difference (p>0.2) in creatinine concentration. Over a period of 31 hours creatinine concentration was stable using enzymatic creatinine assays. A significant increase was seen by 24 hours using Jaffe method (p< 0.025).

The enzymatic assay for creatinine involves a series of coupled enzymatic reactions including creatininase enzymatic conversion of creatinine into the product creatine which is converted to sarcosine by creatinase, followed by oxidation of sarcosine by sarcosine oxidase producing hydrogen peroxide (H_2O_2). In the presence of peroxidase, the hydrogen peroxide is quantified at 546 nm (540-570 nm) by the formation of a red-coloured dye (Marakala *et al*., 2012). Enzymatic creatinine assay is widely accepted as one of the most accurate routine method available at present. The enzymatic method exhibits several advantages over Jaffe's based methods-namely, improved specificity, smaller sample volume and hence rapid sample throughout. Glucose, acetoacetate and cefoxitin do not interfere with the enzymatic method. So, for diabetic ketotic patients, neonates amd patients receiving cephalosporins can be diagnose with the enzymatic method to minimize the errors during measurement of creatinine level. Because principle of Jaffe method is, Creatinine forms a yellow-red colored creatinine picrate complex containing ionic bonds with alkaline picrate solution (in ratio of 1:1). The absorbance of the complex is measured at 492 nm. The rate of formation of the colored complex is directly proportional to the creatinine concentration (Bartel *et al*., 1972). These intermediate substances easily susceptible to interference from non-creatinine chromogens. The presence of interfering substances in serum can lead to over estimation of serum creatinine by as much as 15-25% by various Jaffe methodological applications (Hermida *et al*., 2014). So, creatinine concentration measured by an enzymatic method

are more reliable than the Jaffe method.

Changes in creatinine concentration measured in the refrigerator ($2 - 8°C$) up to 3 days were range from 2.65% to 29.48% (2h to 3 days) and 2.47% to 19.56% (2h to 3 days) by Jaffe and enzymatic methods respectively. When unseparated blood was refrigerated, comparatively the changes were less than the blood stored at room temperature ($p< 0.05$).

V. CONCLUSION

There were no statistical difference ($p> 0.05$) of creatinine concentration that occurred up to 1 day of sample storage by Jaffe method and up to 2 days by enzymatic method. Whole blood sample stored at room temperature ($27 - 29°C$) for serum creatinine measurement by using the Jaffe method is acceptable when samples stored maximum of up to 6 hours only. According to this study, whenever delays occur keeping the blood samples in the refrigerator ($2 - 8°C$) is better than storing at room temperature ($27 - 29°C$). And also measuring serum creatinine by the enzymatic method will minimize the errors than using the Jaffe method and it will lead to the release of accurate test results.

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