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ORIGINAL RESEARCH ARTICLE

Pre-analytical Errors in Glucose Estimation Results in Query on Diabetic Management

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Abstract Status of diabetes of an individual is majorly evaluated by the frequent monitoring of glucose estimation. Use of serum samples and inappropriate plasma for estimating glucose is an existing practise in Indian standard of laboratories. There is a strong evidence for occurrence of in vitro glycolysis on the above mentioned specimens. The aim is to study the pre-analytical variations on the glucose estimation of using sodium fluoride–disodium EDTA (NaF–Na2EDTA) plasma (glycolysis inhibiting anticoagulant) and determine the fact behind the activity of glycolysis inhibition on the same. Healthy volunteers 20–35 years of both genders consisting of 40 members were selected for the study, and after getting the informed consent form, random blood samples were collected to study the errors of pre-analytical i.e., mixing of NaF–Na2EDTA tube by phlebotomist (no of inversion). Difference in duration from blood collection to centrifugation and a variable in time were taken from centrifugation to analyzing the plasma sample. Comparative studies on EDTA plasma and serum sample were also carried out. The usage of the evacuated blood collection system on NaF–Na2EDTA was shown to have the complete glycolysis inhibitor among all pre-analytical errors, whereas other tubes shown considerable increase in glycolysis. Recently the use of glycolysis inhibitor tubes are come into practise only in accredited or certified laboratories. Hence the authentication of glycolysis inhibition study is mandatory for the pre-analytical variations on the same.

Keywords Glycolysis · NaF–Na2EDTA · NABL · ISO

Introduction

Diabetes mellitus (DM) is a complex group of syndromes that cause a common disturbance in the oxidation and utilization of glucose, which is secondary to a malfunction of the beta cells of the pancreas, whose function is the production and release of insulin [1]. Asian population accounts for more than 60% of the world's diabetic patients as the prevalence of diabetes is growing in these countries. Type 2 diabetes mellitus (T2DM) is expected to rise from 285 million in 2010 to 438 million by the year 2030 [2]. T2DM (formerly called non-insulin-dependent or adult-onset diabetes) occurs only in insulin resistance and abnormal beta-cell function involving in polygenic and pathophysiological disorder [3, 4]. American Diabetes Association and the World Health Organization (WHO) proposed the normal values of fasting plasma glucose concentration 126 mg/dl, for random and 2 h post-oral glucose tolerance test concentration $>$ 200 mg/dl [5, 6].

Estimation of blood glucose is mandatory for the identification and management of DM. Glucose oxidase–peroxidase and hexokinase methods are highly standardized with an inter-laboratory imprecision (CV) $<$ 2.6% which was found to be the gold standard enzymatic method for glucose estimation [7–9]. A laboratory error is defined by the quality of laboratory services exaggerated during the

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entire testing process (from ordering tests to reporting results). The most appropriate diagnostic category is critically dependent on the availability of accurate glucose measurements [7, 8].

Pre and post analytical phases are as prominent as the analytical phase frequency of errors [10]. Thus, in glucose measurement, the analytical variation is highly reduced while it is the pre-analytical variation that is responsible for most of total variation in glucose determination. It has been reported an in vitro decrease in plasma glucose samples, not immediately centrifuged, of 5–7%/h due to glycolysis [11]. Most clinical chemistry laboratories will deal with large numbers of glucose specimens per annum, and it is generally acknowledged that a regular sample may take several hours to reach the laboratory; for this reason the prominence of inhibiting glycolysis. Also, samples from general practitioners may travel considerable distances, rendering strict adherence to the WHO recommendations impracticable [12]. Using plasma or whole blood requires the use of an anticoagulant for glucose estimation. The cells do not die immediately when blood is shed or collected because glucose as a source of energy metabolizes and uses up, via the glycolytic process. Glucose thus disappears from whole blood on standing over a period of time. NaF–Na2EDTA inhibits the enzyme enolase which is found in the metabolic pathway of glucose and has little effect on glucose oxidase and peroxidase enzymes. So glycolysis can be prevented with an enzyme inhibitor. NaF–Na2EDTA is the commonest inhibitor for this purpose [10, 13]. Uchida et al. [14] explained in detail of how acidification quickly inhibits glycolysis hexokinase and phosphofructokinase, enzymes inhibited by acidification that act early in the Embden–Meyerhof pathway.

Erythrocytes, leukocytes, and platelets instantly inhibit glycolysis, when the blood pH is maintained between 5.3 and 5.9 with a citrate buffer/EDTA [14]. In addition to immediate centrifugation, glycolysis can be minimized by addition of a glycolysis inhibitor. Granulated additives, present in the NaF–EDTA–citrate tube used are notorious for inducing hemolysis [15] to a large extent; this may be due to improper mixing by phlebotomists [9]. Existing literature study on the pre-analytical variables of proper mixing of tubes, duration taken to centrifuge the samples and the time taken to analyze the blood glucose altogether is a lacuna.

Objective

To study the errors occurring during pre-analytical i.e., mixing of NaF–Na2EDTA tube by phlebotomist (no of inversion), duration taken from bloodshed to centrifugation (immediate to 2 h with 30 min interval), and time taken from centrifugation to analyze the sample (immediate to 2 h with 30 min interval). The comparison is also carried out with EDTA plasma and serum.

Materials and Methods

Forty healthy male and female adults of 20–35 years were selected. The study was briefly explained and received informed consent form from the volunteers, while the blood samples were collected in the outpatient department of Clinical Laboratory of Billroth Hospitals, Shenoy Nagar, Chennai 30. Random blood samples were collected at median cubital vein of left arm by applying tourniquet, from each individual by an experienced phlebotomist using 20 G (BD Vacutainer) directly into 2 ml (NaF–Na2EDTA) vacuum tubes (closed collection system). 2 ml of five tubes were collected from each individual and marked as Serial number followed by an alphabet for e.g., 1a, 1b, 1c, 1d, 1e which refers the time of inversions. (a refers 2 times, b refers 4, c refers 6, d refers 8 and e refers 10 times). As per protocol the samples were centrifuged and plasma glucose were analysed by fully automated Biochemistry analyzer Beckman coulter AU 480, based on the enzymatic analysis of hexokinase at 480 nm.

Categorization

175 samples were grouped into 7, each group consisting of 25 samples. One group was analysed per day.

First Group Consist of 25 Samples Nos. 1–5 of Five Sets

First set of samples were marked as 1a, 2a, 3a, 4a, 5a and inverted for two times and it was processed after duration of < 10 (immediate), 30, 60, 90, 120 min. Time taken from bloodshed to centrifugation immediate (< 10 min). Second set of samples were marked as 1b, 2b, 3b, 4b, 5b and inverted for four times and the remaining procedure was followed as per the previous set. Third set of samples were marked as 1c, 2c, 3c, 4c, 5c and inverted for six times and the remaining procedure was followed as per the previous set. Fourth set of samples were marked as 1d, 2d, 3d, 4d, 5d and inverted for eight times and the remaining procedure was followed as per the previous set. Fifth set of samples were marked as 1e, 2e, 3e, 4e, 5e and inverted for 10 times and the remaining procedure was followed as per the previous set.

Subsequent groups (group 2, 3, 4, 5) were segregated as above said sets. The only difference will be the time taken from bloodshed to centrifugation such as made them to delay as 30, 60, 90 and 120 min, respectively.

GROUP 6—EDTA Plasma of centrifuged 25 samples was collected at 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of 10, 30, 60, 90, 120 min. The time taken from bloodshed to centrifugation was 90 min.

GROUP 7—plain/clot serum of centrifuged 25 samples was collected at 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of 10, 30, 60, 90, 120 min. The time taken from bloodshed to centrifugation was 90 min.

The groups and set are described in the "Appendix".

Statistics Analysis

Data were analysed using SPSS-16. Statistical comparison were made using one way analysis of variance (ANOVA) and followed by Duncan post hoc. Results are presented as mean ± SEM (Standard Error Mean). A probability level (p) of < 0.05 (5%) or less were considered no statistically significant.

Results

The results obtained in the experimental study as represented in Figs. 1, 2, 3, 4 and 5 revealed no significant alterations in plasma glucose levels determined in all five groups of NaF–Na2EDTA plasma. Comparison within and between five sets of samples at different inversion time (2 time vs. 4, 6, 8, 10 times), different centrifugation time [< 10 (immediate), 30, 60, 90, 120 min] and different processing time [immediate ~ < 10 (immediate), 30, 60, 90, 120 min] showed no significant changes were observed on all the NaF–Na2EDTA plasma samples. Whereas the results obtained in the experimental study as depicted in Fig. 6 revealed significant alteration ($p < 0.05$) in plasma glucose levels determined in all five groups of EDTA tube and clotting tube samples. Comparison within and between five sets of samples at different inversion time (2 time to 4, 6, 8, 10 times), and processed at different time intervals of 10, 30, 60, 90, 120 min and centrifugation time of 90 min showed significant changes in the occurrence of glycolysis.

Each bar represents mean ± SEM of 10 sets statistical significance at $p < 0.05$. Each first bars indicates EDTA inversion times. Each second bar indicates plain sample inversion times, represented in rectangles showing significant difference between EDTA and serum. The rectangles visualized the EDTA alone increased at 6 and 10 time inversions compared with serum at 6 and 10 time inversion. It rapidly decreased due to inhibition of glycolysis which takes place alternatively while the serum alone increased at 4 and 6 time inversions compared with EDTA at 4 and 6 time inversion which rapidly decreased due to glycolysis taking place. The above results show pre-analytical errors such as improper mixing and delay to centrifugation and processing impact glucose estimation when compared with NaF–Na2EDTA.

First set of samples were represented as *a, b, c, d* for EDTA and serum $a^{\$}$, $b^{\$}$, $c^{\$}$, $d^{\$}$ for serum and inverted for 2, 4, 6, 8, 10 times and it was centrifuged at 90 min processed after duration of < 10 (immediate), 30, 60, 90, 120 min.

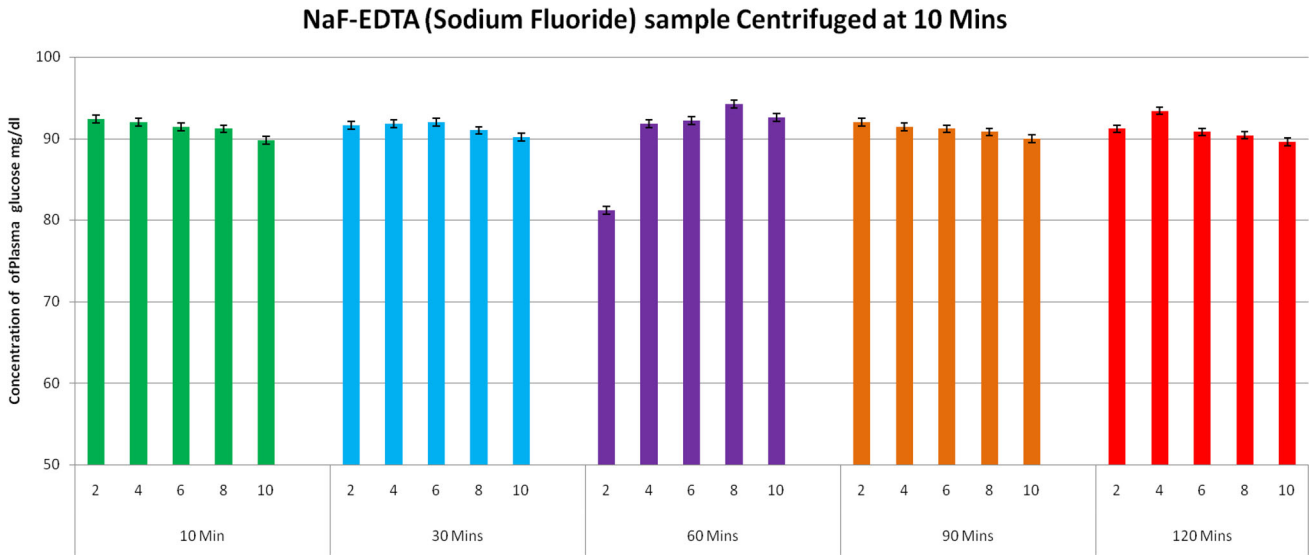

Fig. 1 Blood glucose level in all the five sets of plasma sample centrifuged at 10 min (n = 25). The glucose concentration determined in NaF–EDTA plasma of < 10 min immediate centrifuged 25 sample was collected as 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of 10, 30, 60, 90, 120 min. Each bar represents mean ± SEM of five sets non-statistical significance at $p < 0.05$

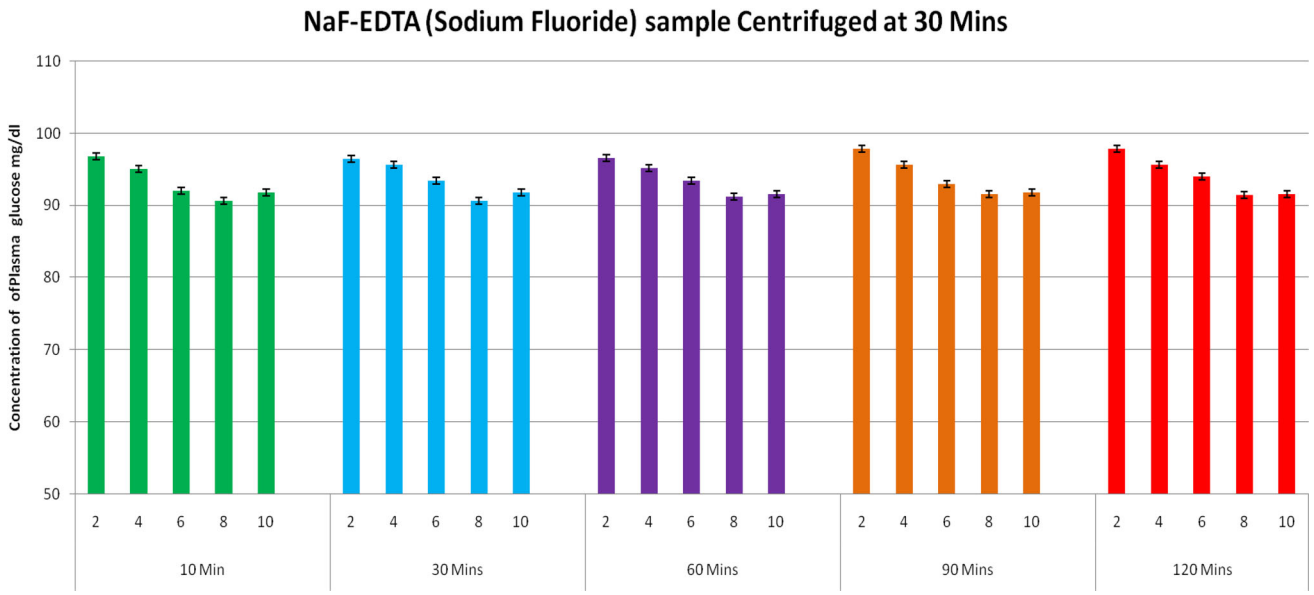

Fig. 2 Blood glucose level in all the five sets of plasma sample centrifuged at 30 min (n = 25). The glucose concentration determined in NaF–EDTA plasma of after 30 min centrifuged 25 sample was collected as 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of 10, 30, 60, 90, 120 min. Each bar represents mean ± SEM of five sets non-statistical significance at $p < 0.05$

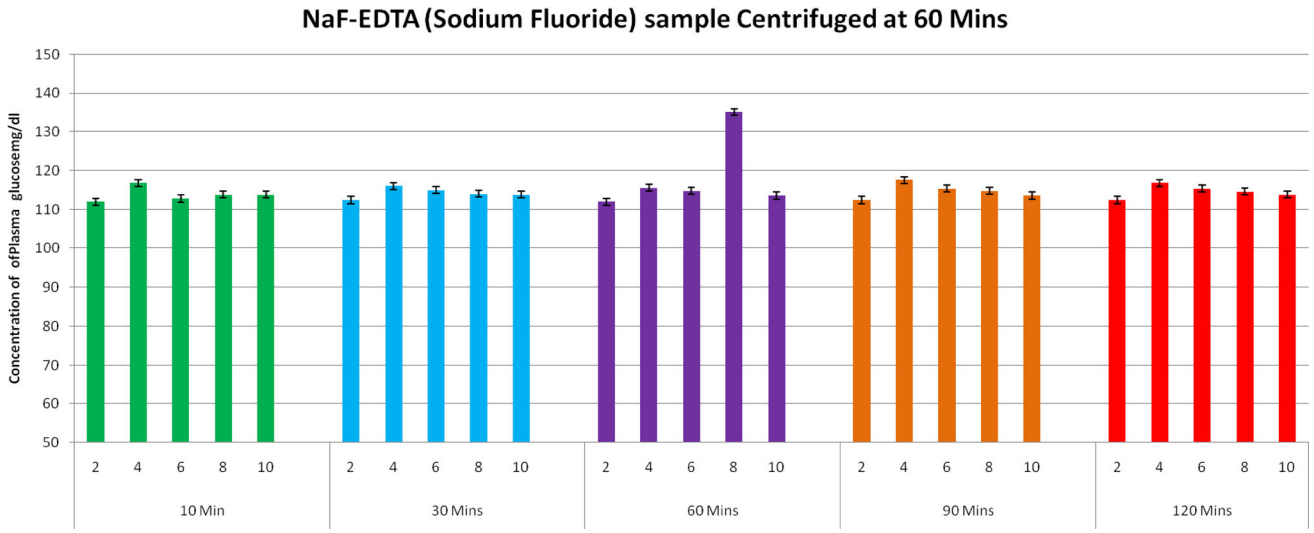

Fig. 3 Blood glucose level in all the five sets of plasma sample centrifuged at 60 min (n = 25). The glucose concentration determined in NaF–EDTA plasma of after 60 min centrifuged 25 samples was collected as 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of 10, 30, 60, 90, 120 min. Each bar represents mean ± SEM of five sets non-statistical significance at $p < 0.05$

Subsequent sets of 10, 30, 60, 90, 120 min were segregated as above said sets. Similar procedures for serum and EDTA inverted for 2, 4, 6, 8, 10 times and it was centrifuged at 90 min. The only difference will be the time taken for processed after $\sim <$ 10, 30, 60, 90, 120 min. It will represented by the symbols.

Discussion

Laboratory diagnosis plays a major role in the management of disease and the treatments follow-up. There are nearly 10,000 laboratories in Chennai among them only 35–40 got accredited with national accreditation according to International organisation for standardization (ISO) standards. It usually becomes the primary activity of a handful of persons in the laboratory who create a bureaucracy for the purpose and some got accredited from College of American Pathologist (CAP) and only 3 received National

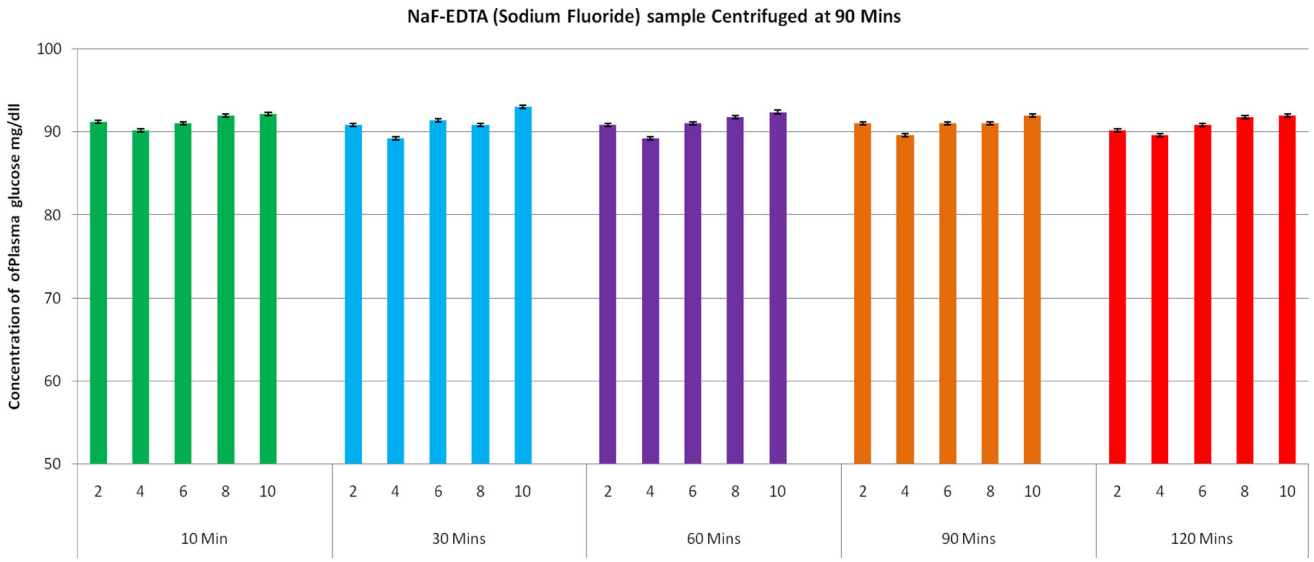

Fig. 4 Blood glucose level in all the five sets of plasma sample centrifuged at 90 min (n = 25). The glucose concentration determined in NaF–EDTA plasma of after 90 min centrifuged 25 samples was collected as 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of 10, 30, 60, 90, 120 min. Each bar represents mean ± SEM of five sets non-statistical significance at $p < 0.05$

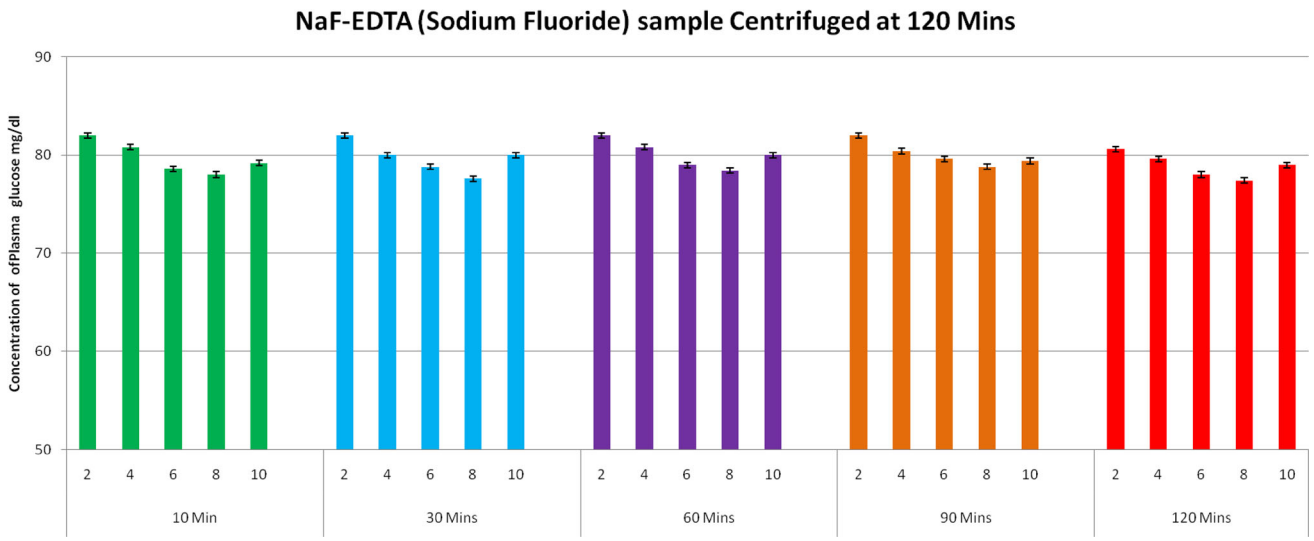

Fig. 5 Blood glucose level in all the five sets of plasma sample centrifuged at 120 min (n = 25). The glucose concentration determined in NaF–EDTA plasma of after 120 min centrifuged 25 samples was collected as 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of 10, 30, 60, 90, 120 min. Each bar represents mean ± SEM of five sets non-statistical significance at $p < 0.05$

Accreditation Board for Hospitals & Healthcare Providers (NABH) med lab. Standards and accreditation are important for quality assurance but in their basic nature they strive for status quo rather than for dynamic development with the inherent risks that invite changes. However, the people involved in both laboratories and their accreditation authorities are afraid that the process of change decreases quality.

Quality indicators can be defined as objective measures developed and implemented to assess any critical health care part such as patient safety [16, 17]. Testing cycles outlined a chain of activity, spot on from arising of doubt in the clinician's mind, test selection, sample collection, sample transportation to the laboratory, sample investigation, interpretation of reports and finally decision making by the clinician [18]. Diagnosis, management, treatment of patients and eventually patient safety itself can be compromised by poor quality of collection methodology that is suggesting procedure for diagnostic specimen collection, which is still not completely adopted by the existing

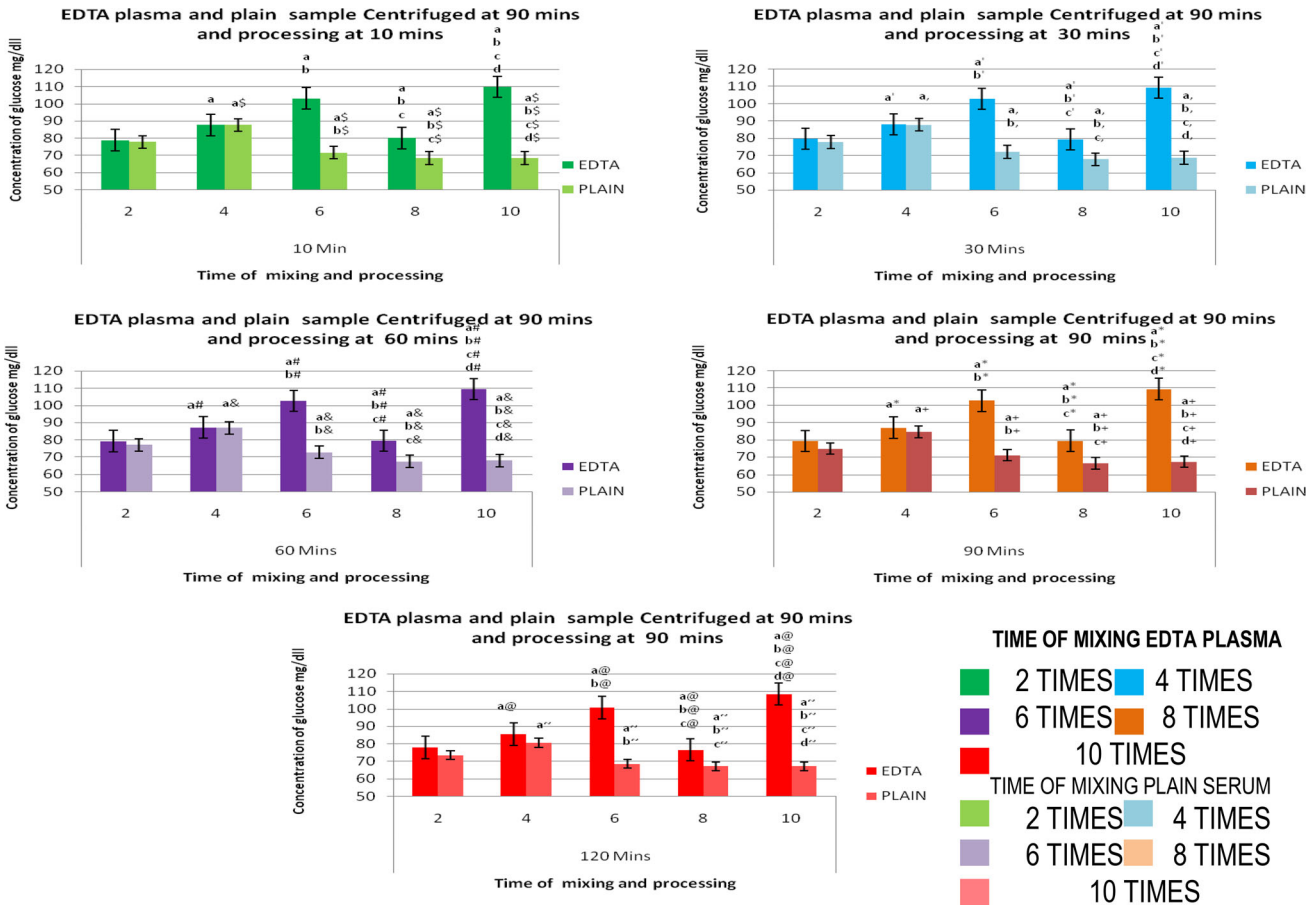

Fig. 6 Blood glucose level in all the five sets of EDTA plasma sample and plain serum centrifuged at 90 min versus time of mixing and processing (n = 50)

clinical laboratories. Therefore there is large diversity in sample collection procedures. Various modes of venous blood specimen collection methodology and devices are used. Closed evacuated blood collection system is being used by only in certified laboratories, while majority use conventional needles and syringes to take blood and very few use non vacuum tubes. The influence of such practices on quality of specimens has not been established in the past. We find out the impact of different modes of venous blood collection on various pre-analytical specimen used for glucose estimation. Increased focus on achieving better analytical quality through proper usage of improved quality of collection methodology and devices are used to achieve total quality of results [19].

The pre-analytical study of Kume [20] determined the most common problems as follows: the syringes used as an alternative of a vacuum system for blood collection which causes insufficient fill lines, haemolysis as a result of collection by needles with small diameters, hemolysis because of blood transfer from needle to vacuum tubes without discarding the needle from the syringe and clotted samples because of insufficient mixing of anticoagulant and blood. Özcan and Güreser [21] reported 73% of errors occurred due to improper blood collection.

Diagnostic blood specimens subsequent to collection mixing with suitable additives without delay were efficiently important, and all vacuum tubes manufacturers' are also recommended by datasheets and CLSI documents. Parenmark and Landberg [22] recently released an influential emulation about the mixing procedure of the diagnostic blood specimens. Instant mixing blood samples after collection need not be obligatory for all types of tubes; and instant mixing may produce false hemolysis and thereby initiate a bias for those parameters that are most susceptible to RBC damage [23–25]. The more reasonable explanation for in our study impact of improprius inversion of turmoil generated NaF–EDTA sample for (2, 4, 6, 8, 10 times) was monitored to assess its effect on glycolysis inhibition.

Although there are guidelines by CLSI document suggestive of procedure for diagnostic specimen collection, there is large diversity in sample collection procedures that are not obligatory in India. The country used various modes of venous blood specimen collection methodology and devices. Closed evacuated blood collection system is being

used by only small number of users, while mass use of needles and syringes and transfer blood to re-used glass vials or very few use non vacuum tubes. In the past, influence of such practices on quality of specimens has not been established. Unfortunately, Melkie [26] systematize training and continuous education plans worldwide or else did not stratify the regularity of disagreeable mixing of specimens from blood collection using a vacuum system or a syringe and needle it has considered by directors, quality managers and laboratory. Other preservatives used to prevent glycolysis, e.g., glyceraldehyde 3 phosphate dehydrogenase iodoacetate, which inhibits, also takes up to 3 h to become effective [27]. Uchida et al. [14] describes a more effective method of glycolysis inhibited by acidification of blood. Acidification inhibits hexokinase and phosphofructokinase enzymes that act early in the glycolytic pathway. Acidification is sustainable inhibitory effect for approximately 8 h at 25 °C. This study is purposefully intended to assess errors in the mixing of the tube, delay in centrifugation and delay in the analyzing the sample.

The results obtained clearly depicted that there is no statistically significance seen in the improper mixing of 2, 4, 6, 8 and 10 times at different time intervals (< 10, 30, 60, 90, and 120 min) of centrifugation and processing in all five groups processed with NaF–Na2EDTA plasma sample. NaF–Na2EDTA acts as a complete glycolysis inhibitor which maintains the plasma glucose and remains stable in all the improper mixing, centrifugation and processing delayed samples. Whereas significant variations occurs in glucose levels was observed in group six (EDTA plasma and plain serum) samples mixed for 2, 4, 6, 8, 10 different times inversion and processed at different time intervals of < 10, 30, 60, 90, 120 min. The time taken from bloodshed to centrifugation was 90 min. Pre-analytical variables such as the choice of phlebotomy material and post-phlebotomy turn-around-time (TAT) affect in vitro glucose stability [27]. In vitro, glucose levels may drop as much as 7%/h (± 0.6 mmol/l/h) due to ongoing glycolysis due to it does not prevent a drop during the first hours after phlebotomy [10, 28]. Even though TAT was typically longer than 60 min, most laboratories undertook no action [29]. Aforementioned existing literatures nonetheless focusing on TAT such as longer than 60 min for glucose estimation, and also In vitro, glucose levels dropped percentage in hours. Based on these studies we decided to focus pre-analytical variables such as the choice of phlebotomy material (plain, EDTA tubes and syringes-open collection system) and post-phlebotomy turn-around-time (TAT) is 90 min fixed to analyze in vitro glycolysis. The EDTA concentration alone increased at 6 and 10 time inversions compared with serum at 6 and 10 time inversion. This was rapidly increased due to inhibition of glycolysis which takes place alternatively. While the serum concentration alone increased at 4 and 6 time inversions compared with EDTA at 4 and 6 time inversion which rapidly decreased due to glycolysis taking place. The levels of serum glucose may vary in delaying of analysis, which is consistent with delay of transport of samples from the collection centres to the central laboratory [13, 30]. Serum and EDTA plasma glucose levels were significantly altered as compared to NaF–Na2EDTA. Results of the study emphasize that NaF–Na2EDTA tube is the only tube which inhibits the complete glycolysis, even impact of pre-analytical errors which proves the myth of the accredited lab professional as quoted by Orwell—*"Myth which are believed in tend to become true"*. Resources of non-accredited lab professional diagnosing diabetic patients should have awareness about quality of laboratory testing in glucose estimation.

Conclusion

Pre-analytical errors are largely accountable to human mistakes and the majority of these errors cannot be escapable. True plasma glucose level determination is important not only for diagnosis of diabetes but also to identify high risk patients effectively. Evacuated closed blood collection with vaccutainer is improving the quality of laboratory testing while compared to open collection system by using a needle and syringe. No statistical significance was observed in the glucose levels measured from NaF–Na2EDTA plasma tubes used to assess the errors in the mixing of the tube, delay in centrifugation and delay in the analyzing the sample. They showed non-significance $p > 0.05$ in glucose levels indicating the inhibition of glycolysis. Whereas EDTA and serum sample showed significance $p < 0.05$ in glucose levels clearly indicating the occurrence of glycolysis. This study suggests that health care professionals, clinical laboratory professionals and patients in diabetic management should have awareness about quality of laboratory testing in glucose estimation. To select the right lab, right blood collection systems, right methodology will provide the perfect diagnosis which can be trust worthy and dependable for the management of any deficiency and diseases. If it fails, it paves the pathway for improper management of diabetes.

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Appendix: Table For Mixing Delay, Centrifugation Delay And Analysing Delay

Group 1

No samples	No of time inverted		Time taken from completion of centrifuge to analyze				
			< 10 min	30 min	1.00 h	1.30 h	2.00 h
1a, 2a, 3a, 4a, 5a	2	Time taken from blood shed to centrifuge					
1b, 2b, 3b, 4b, 5b	4	Immediately centrifuge (< 10 min)					
1c, 2c, 3c, 4c, 5c	6						
1d, 2d, 3d, 4d, 5d	8						
1e, 2e, 3e, 4e, 5e	10						

Sample ID will be designated in serial nos. 1–5 the same samples will be taken for variable time of mixing

Group 2

No samples	No of time inverted		Time taken from completion of centrifuge to analyze				
			< 10 min	30 min	1.00 h	1.30 h	2.00 h
6a, 7a, 8a, 9a, 10a	2	Time taken from blood shed to centrifuge					
6b, 7b, 8b, 9b, 10b	4	Immediately centrifuge (30 min)					
6c, 7c, 8c, 9c, 10c	6						
6d, 7d, 8d, 9d, 10d	8						
6e, 7e, 8e, 9e, 10e	10						

Sample ID will be designated in serial nos. 6–10 the same samples will be taken for variable time of mixing

Group 3

No samples	No of time inverted		Time taken from completion of centrifuge to analyze				
			< 10 min	30 min	1.00 h	1.30 h	2.00 h
11a, 12a, 13a, 14a, 15a	2	Time taken from blood shed to centrifuge (1.00 h)					
11b, 12b, 13b, 14b, 15b	4						
11c, 12c, 13c, 14c, 15c	6						
11d, 12d, 13d, 14d, 15d	8						
11e, 12e, 13e, 14e, 15e	10						

Sample ID will be designated in serial nos. 11–15 the same samples will be taken for variable time of mixing

Group 4

No samples	No of time inverted		Time taken from completion of centrifuge to analyze				
			< 10 min	30 min	1.00 h	1.30 h	2.00 h
16a, 17a, 18a, 19a, 20a	2	Time taken from blood shed to centrifuge (1.30 h)					
16b, 17b, 18b, 19b, 20b	4						
16c, 17c, 18c, 19c, 20c	6						
16d, 17d, 18d, 19d, 20d	8						
16e, 17e, 18e, 19e, 20e	10						

Sample ID will be designated in serial nos. 16–20 the same samples will be taken for variable time of mixing

Group 5

No samples	No of time inverted		Time taken from completion of centrifuge to analyze				
			< 10 min	30 min	1.00 h	1.30 h	2.00 h
21a, 22a, 23a, 24a, 25a	2	Time taken from blood shed to centrifuge (2.00 h)					
21b, 22b, 23b, 24b, 25b	4						
21c, 22c, 23c, 24c, 25c	6						
21d, 22d, 23d, 24d, 25d	8						
21e, 22e, 23e, 24e, 25e	10						

Sample ID will be designated in serial nos. 21–25 the same samples will be taken for variable time of mixing

Group 6 (EDTA Sample)

No samples	No of time inverted		Time taken from completion of centrifuge to analyze				
			< 10 min	30 min	1.00 h	1.30 h	2.00 h
26a, 27a, 28a, 9a, 30a	2	Time taken from blood shed to centrifuge (1 h .30 min)					
31b, 32b, 33b, 34b, 35b	4						
36c, 37c, 38c, 39c, 40c	6						
41d, 42d, 43d, 44d, 45d	8						
46e, 47e, 48e, 49e, 50e	10						

Sample ID will be designated in serial nos. 1–5 the same samples will be taken for variable time of mixing

Group 7 (Serum Sample)

No samples	No of time inverted		Time taken from completion of centrifuge to analyze				
			< 10 min	30 min	1.00 h	1.30 h	2.00 h
51a, 52a, 53a, 54a, 55a	2	Time taken from blood shed to centrifuge (1 h .30 min)					
56b, 571b, 58b, 59b, 60b	4						
61c, 62c, 63c, 64c, 65c	6						
66d, 67d, 8d, 69d, 70d	8						
71e, 72e, 73e, 74e, 75e	10						

Sample ID will be designated in serial nos. 1–5 the same samples will be taken for variable time of mixing

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