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SHORT COMMUNICATION

Comparison of Citrate Buffer with Sodium Fluoride as Additives in Determining Glycemia

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SUMMARY

Background: **The objectives of this study are to compare the effect of sodium fluoride and citrate on the stability of glucose in samples maintained at room temperature up to three hours, and to assess the clinical impact in the O'Sullivan test results after changing the additives in the collecting tubes.**
Methods: **The selected population was pregnant women between the 24th and 28th week of gestation, who were at the health center to undergo the O'Sullivan test as part of the screening program for GDM (gestacional diabetes mellitus). Two blood samples were extracted from each patient: one using a tube with citrate and sodium fluoride buffer (tubes Vacuette Glucomedics citrate, 2 mL, Ref 454347) (tube C) and another containing just sodium fluoride (BD Vacationer tubes FX fluoride, 2 mL, Ref 368920) (tube F).**
The statistical treatment of the data was performed using SPSS version 24 and Method validator. Finally, we assessed the real clinical impact of replacing tubes C for tubes F in the classification of pregnant women. To do so, we collected the results of O'Sullivan tests conducted in our hospital during a year, all of them done in tubes F, and we applied the mean difference calculated in T = 1 to estimate the number of pregnant women that should be reclassified.
Results: **The average glycaemia in tubes C are significantly greater than average glycaemia in tubes F ($p < 0.05$) at all time points. The clinical impact assessment was done over the 6,526 O'Sullivan test results with a prevalence of positive tests of 21.35%. The prevalence using tubes C instead of tubes F estimated with mean differences previously calculated is 33.45%.**
Conclusions: **The glucose concentrations in tubes F stored at room temperature up to 3 hours were significantly lower ($p < 0.05$) than those measured in tubes C stored under the same conditions. We observed that it is in the first minutes after extraction, while the samples are collected and aliquots done, that the glucose consumption occurs in tubes F, but not in tubes C. There is a need to change the preanalytical conditions to prevent any loss of glucose. This will enable more accurate diagnosis and management of diabetes mellitus.**
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KEY WORDS

sodium fluoride, citrate buffer, glycolysis inhibitor, diabetes mellitus

INTRODUCTION

Laboratory plasma glucose testing is essential for the diagnosis of glucose metabolism disorders and, most of all, for the screening and diagnosis of gestational diabetes mellitus where glycated hemoglobin cannot be used

[1-3]. Therefore, it is clear that plasma glucose measurement must be accurate to correctly classify patients according to international guidelines [4].

The loss of glucose in blood samples has been studied for many years [5]. Glucose is consumed in glycolysis at a rate of 5 - 7% per hour at concentrations near the reference interval. The highest loss rates occur in samples with high white blood cell counts [6] and with increasing ambient temperature.

The principal analytical methods used for glucose determination are enzymatic assays, mainly based on hexokinase, although glucose oxidase reaction is also used [5]. These methods are highly standardized with an interlaboratory imprecision (CV) < 2.6% [1,4,5]. Accordingly, the analytical variation is highly reduced while there is preanalytical glucose consumption during the first 1 - 2 hours after blood collection, consequently this is a much larger source of error than the analytical error [4-7].

In 1923, Major introduced potassium fluoride as a potent inhibitor of glycolysis, and it has been used for decades, usually as NaF, to inhibit glycolysis in blood samples. However, it has been proven [6] that NaF has little or no effect on the rate of glycolysis during the first 1 - 2 hours or more after blood is collected. Such findings appear to reflect the fact that the enzyme inhibited by fluoride, enolase, is the one that catalyzes reactions at the end of glycolysis. Therefore, until the chemical equilibrium is reached in the reactions close to this, glucose is further metabolized.

In recent years, the handling of blood samples collected for analysis of glucose has been little studied, perhaps reflecting the mistaken belief that the use of NaF had resolved the preanalytical problems.

To minimize *in vitro* glycolysis, the American Diabetes Association (ADA) and the National Academy of Clinical Biochemistry (NACB) recommend to place the sample tube immediately in an ice-water slurry and to separate plasma from cells within 30 minutes [8]. However, this is not a practical solution in today's health care, with health centers organized to send the samples to the central laboratory, often located up to three hours away. Gambino et al. [9] have reported that the stability of glucose in ice slurry can be achieved using a blood-collection tube that was described in a US patent more than 20 years ago, but is little known in most laboratories. The additive in these tubes is a citrate buffer: acidification of blood pH below 5.9 ensures faster inhibition of glycolysis and inhibiting hexokinase and phosphofructokinase, enzymes involved in the first steps of glycolysis [10].

The O'Sullivan test is used in the Andalusian health service program of pregnancy, childbirth, and postpartum for gestational diabetes screening. The blood collection for the O'Sullivan test is carried out in health centers, and transport of samples from health centers to the central laboratory usually takes from 1 to 3 hours.

The objectives of this study are, on the one hand, to compare the effect of sodium fluoride and citrate on the stability of glucose in samples maintained at room temperature up to three hours, and, on the other hand, assess the clinical impact in the O'Sullivan test results after changing the additives in the collecting tubes.

MATERIALS AND METHODS

Population and samples

The selected population was pregnant women between the 24th and 28th week of gestation, who were at the health center to undergo the O'Sullivan test as part of the screening program for gestational diabetes. We calculated the required sample size based on a preliminary test performed with five samples, which proved n = 48 to be sufficient to have statistical significance.

Patients were informed of the study and signed the informed consent. A first blood sample was extracted for measurement of fasting glucose. After 60 minutes of ingesting 50 g of glucose two blood samples were extracted from each patient: one using a tube with citrate and sodium fluoride buffer (tubes Vacuette Glucomedics citrate, 2 mL, Ref 454347) (tube C) and another tube containing just sodium fluoride (BD Vacutainer tubes FX fluoride, 2 mL, Ref 368920) (tube F).

Each tube was divided into four aliquots which were successively centrifuged at 3,500 rpm for 10 minutes. The first two aliquots (one from tube C and one from tube F) were centrifuged immediately after extraction (T = 0), second two after one hour (T = 1), the third after two hours (T = 2), and the fourth after three hours (T = 3). Glucose was determined in each sample aliquot on a Siemens analyzer ADVIA®2400 using the glucose hexokinase method. The analyzer is controlled daily before the start of the routine work using quality controls MULTIQUAL CONTROL (Level 1, 2, 3) BIO-RAD®.

Statistical analysis

We collected the results of glycemia obtained in a database. The statistical treatment of the data was performed using SPSS version 24 and Method validator. The Shapiro-Wilks test was used to determine the normal distribution of data. We calculated the average glycemia for tube F and C at each time point, the results were expressed as mean ± standard deviation (SD). We used Student's *t*-test to establish the statistical significance of the differences between glucose measured in tube F and C. The differences were also analyzed with the Bland Altman plot.

Clinical impact assessment

Finally, we assessed the real clinical impact of replacing tubes C for tubes F in the classification of pregnant women. To do so, we collected the results of O'Sullivan tests conducted in our hospital between September 2014 and September 2015, all of them done in tubes F, and we applied the mean difference calculated in T = 1 to estimate the number of pregnant women that should be reclassified if tubes F were replaced by tubes C. We compared the results obtained using box plots.

Table 1. Average glycemia (mean and standard deviation, SD) obtained at each time point (0 to 3) for all the samples in both types of tubes, and minimum and maximum values of glucose.

Time (hours)	Glycemia (mg/dL) in tube C				Glycemia (mg/dL) in tube F			
	Mean	Minimum	Maximum	SD	Mean	Minimum	Maximum	SD
0	128	81.2	379.3	47.8	117.1	68	328	42.1
1	128.6	83.5	375.8	48.2	115.6	67	324	41.8
2	128.7	80.04	353.8	44.2	114.5	70	327	41.8
3	128.3	80.04	349.2	43.3	113.7	72	324	42.2

Table 2. Glycemia (in mg/dL) mean differences (Mean) between tube C and tube F at each measurement time; Standard Deviation (SD) of that mean differences; mean difference 95% confidence interval (95% CI); t-score obtained for paired samples data (t-score); and statistical significance (p-value) of differences.

Time (hours)	Differences Glycemia Tube C - Tube F (mg/dL)				Student's *t*-test	
	Mean	SD	95% CI		t-score	p-value
0	10.87	8.98	8.27	13.48	8.389	0.000
1	12.89	5.24	11.37	14.42	17.051	0.000
2	14.79	7.08	12.74	16.85	14.481	0.000
3	14.54	5.89	12.83	16.25	17.108	0.000

Table 3. Pregnant population attended between September 2014 and September 2015 and positive O'Sullivan test results (glycemia ≥ 140 mg/dL 1 hour after 50 grams glucose intake) using tube F and the same results estimated for tube C.

Number of tests		6,526	
Age		32.07 (± 5.47) years	
Gestational age		24 - 28 gestational weeks	
Tube F		Tube C	
Positives	Prevalence	Positives	Prevalence
1,393	21.35%	2,183	33.45%

RESULTS

Figure 1 shows the differences between mean glycemia measured in each tube. Table 1 shows detailed results for the average glycemia measured in both kinds of tubes in the 48 samples and at each of the four different time points, with minimum and maximum values and standard deviations (SD). The differences between mean glycemia measured in each tube can be visualized in Figure 1.

First, we proved normal distribution of the data using the Shapiro-Wilks test, and then we used paired samples Student's *t*-test to determine the statistical significance of the differences. As we show in Table 2, the average glycemia in tubes C are significantly greater than average glycemia in tubes F ($p < 0.05$) in all times. Figure 2 shows the Bland-Altman plots for each time.

The clinical impact assessment was done using the O'Sullivan test results conducted in our hospital between September 2014 and September 2015 (Table 3). The number of tests were 6,526 with a prevalence of positive tests (glycaemia ≥ 140 mg/dL 1 hour after 50 grams glucose intake) of 21.35%. The prevalence using C tubes instead of F tubes estimated with mean differences previously calculated is 33.45%. Then we obtained box plots to compare the different O'Sullivan test results using each type of tube (Figure 3).

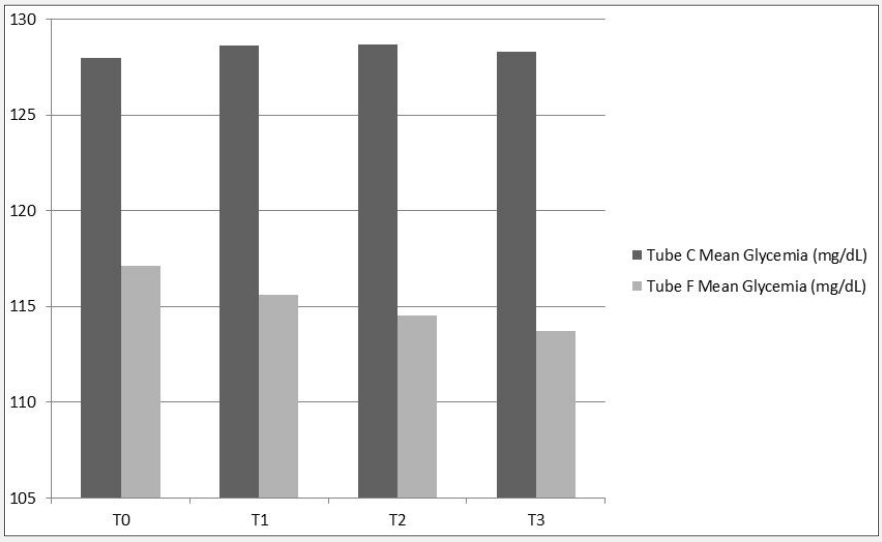

Figure 1. Mean glycemia in each tube in mg/dL at each time point (in hours).

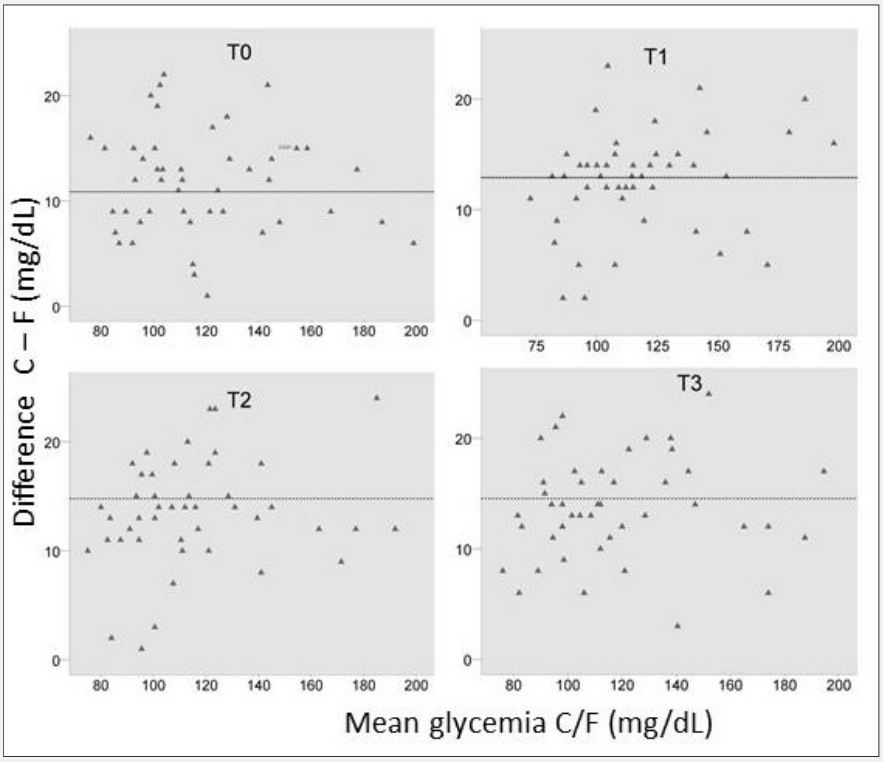

Figure 2. Bland-Altman plots, differences (tube C - tube F) in mg/dL versus mean glycemia in mg/dL for each time (T = 0 hour, 1 hour, 2 hours, and 3 hours).

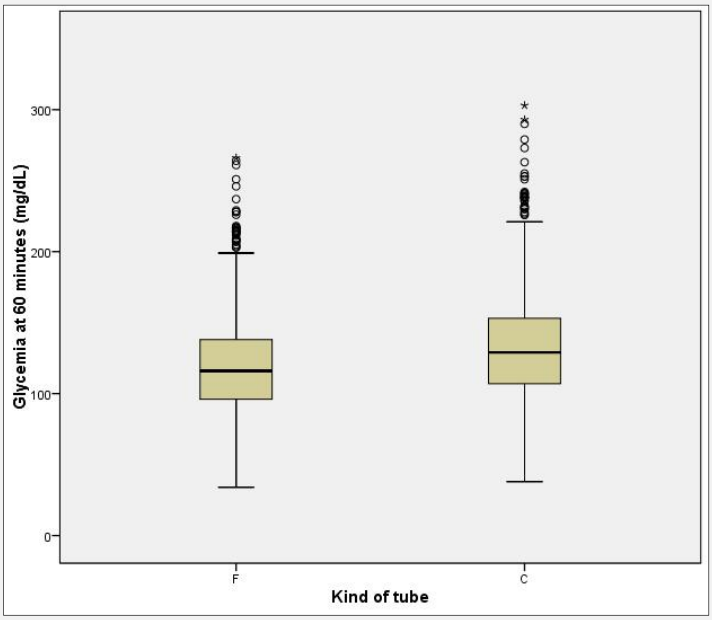

Figure 3. Box plot showing the distribution of O'Sullivan test results observed in our hospital between September 2014 and September 2015 in F tubes (F), and distribution of O'Sullivan test results estimated in the same period if C tubes would have been used instead of F tubes (C).

DISCUSSION

The glucose concentrations in F-tubes stored at room temperature up to 3 hours were lower than those measured in C-tubes stored under the same conditions. The results indicated that blood glucose means in C-tubes were significantly higher ($p < 0.05$) than the mean blood glucose levels in F-tubes.

When analyzing the results, we observed that it is in the first measurement ($T = 0$) that there is a greater variation in blood glucose, with a decrease of 8.6% in blood glucose in F tube with respect to C tube and that these are stabilized in the following measurements in both tubes. Our results confirm that citrate buffer blocks glycolysis at an earlier stage than NaF, as previously reported [11,12].

These data are in line with the study by Gambino et al. [9], where they prove that the C-tubes allow the maintenance of a glucose concentration that coincides with that obtained with the reference method, even after 4 hours of storage.

We have to emphasize that after analyzing the results, we observed that the first hour of storage is crucial in glucose integrity, and, therefore, the additive used in the extraction tube is critical not only for the samples coming from distant Health Centers, but in all of the Reference Area. C-tubes ensure a faster and more efficient glycolysis inhibition to allow glucose concentration integrity at the time of determination in the laboratory, requiring the replacement of the current extraction tube for the O'Sullivan Test in the Primary Care Centers. The clinical impact assessment was done using the O'Sullivan test results conducted in our hospital between September 2014 and September 2015. The number of tests was 6,526 with a prevalence of positive tests of 21.35%. After applying the average difference of an 8.6% increase in blood glucose, corresponding to 1 hour post-extraction, to the O'Sullivan Test results from our health area, we obtained that there would be a 12.1% increase in the number of positive tests.

In the epidemiological studies from which the cutoff points for the diagnosis of diabetes mellitus were defined, it is not always clear which type of additive has been used [13,14]. Thus, our study together with others recently published [15-17], suggest the need for new standardized studies using C tubes, in order to know the glycemic levels of the population and to consider the need to redefine the cutoff points for diabetes mellitus.

CONCLUSION

There is a need to change the preanalytical conditions to prevent any loss of glucose by glycolysis in samples for glucose estimation. This will enable estimation of true glucose levels and thus accurate diagnosis and management of diabetes mellitus.

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Declaration of Interest:
The authors declare that they have no conflicts of interest.

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