DOI: 10.4081/aiua.2024.12186

ORIGINAL PAPER

Examination of ejaculate fructose levels on male infertility patients at various times and centrifugation using semiautomatic method

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Summary *Objective: Various factors, such as obstructive azoospermia, cause infertility in men. Biochemical examination of ejaculate, especially measurement of fructose, can be an additional investigation that can be used for this diagnosis in reproductive health. Examination of fructose is carried out after routine ejaculate analysis, resulting in prolonging the examination time so that it will affect the measurement of fructose level in the ejaculate and the accuracy of the diagnosis. This study aims to determine the best timing and procedure for measurement of fructose using a semiautomatic method.*

Methods: This research is an analytic observational study conducted at Dr. Soetomo General Hospital, Surabaya. A total of 13 ejaculate samples from infertile male patients who met the inclusion criteria were evaluated. Each ejaculate was divided into eight aliquots that were examined for fructose using a semiautomated method after different intervals of time and centrifugation modalities.

Results: This study showed a significant difference in fructose levels when aliquots were centrifuged and examined immediately or after different interval of time ($p = 0.036$). In addition, aliquots left standing for more than 60 minutes ($p = 0.012$) and 120 minutes ($p < 0.001$) before centrifugation, showed significantly lower levels compared to aliquots that were centrifuged and then immediately examined.

Conclusions: We suggest that measuring fructose immediately after centrifugation is more reliable than measuring fructose left standing before or after centrifugation. Leaving the ejaculate standing will reduce the fructose level so that it does not resemble its real level.

KEY WORDS: *Infertile; Fructose; Semiautomatic; Time; Centrifugation; Reproductive health.*

Submitted 13 December 2023; Accepted 4 January 2024

INTRODUCTION

The fructose examination is one of the biochemical tests used to diagnose the cause of male infertility (1). It is not a routine test, but it is a useful supplemental test to provide information on specific clinical conditions, that is done after routine testing ejaculate analysis (2). According to *World Health Organization* (WHO) ejaculate analysis laboratory guidelines 2021, each standard ejaculate analysis test takes about 60 minutes, so the timing to test fructose is extended, which may affect the measurement of the level of fructose in ejaculate (3). In fact, when fructose is tested in this way, the fructose level obtained will differ from the real fructose level because the fructose in the ejaculate will undergo fructolysis. Nonspecific and sensitive test results will inevitably influence the diagnosis.

There are several methods to test for fructose, including colorimetric, enzymatic, and chromatographic methods (4). WHO used Karvonen and Malm's modified methods to test the amount of fructose in ejaculation (3). The manual fructose testing method has disadvantages, such as a complicated procedure with a long waiting time. Since 1981, the use of semiautomatic or automatic machines for testing fructose began to develop (5). The semiautomatic machine can shorten the time to test the fructose level. The advantages of the semiautomatic fructose method include easy reagent preparation, minimal reagent use, minimal error, reduced human resources, and easy calibration and quality control processes (6).

There is no standardized time and method for semi-automated fructose testing, so the researchers felt it was necessary to research to find the best timing for testing fructose in order to obtain results close to real fructose levels. In this study, we carried out the process using a BTS-350 semiautomatic machine. The method used was an enzymatic method using hexokinase and phosphoglucoisomerase enzymes and then switching to a colorimetric method. The method used in BTS-350 semiautomatic machines makes the processing time much faster.

METHODS

This was an observational analytic study using the ejaculates of infertile men who visited the *Policlinic Andrology Outpatient General Hospital, Dr. Soetomo Surabaya*.

The study was approved by the ethics committee of *RSUD Dr. Soetomo* with no. 0669/KEPK/V/2023.

Based on the sample calculation, 13 ejaculates from different men were included in this study.

Inclusion criteria were samples from men aged 18-50 years old, with ejaculate volume > 2 ml, and willing to participate in the study by signing informed consent; we exclude men with anejaculation, haematospermia, hyperviscosity, and men with reproductive infection.
Each man who meets the criteria was explained about the procedures and objectives of the study and signed an informed consent if he was willing to participate to the research with his sample. Then, the participant was asked to masturbate and collect ejaculate into a non-toxic container.
After that, each ejaculate was divided into eight aliquots which were managed in different ways after ejaculate liquefaction. K1: the aliquot was immediately examined for fructose content; K2: the aliquot was centrifuged, then the supernatant was taken, and fructose content was examined; K3: the aliquot was centrifuged and allowed to stand for 30 minutes, then the supernatant was taken and examined for fructose; K4: the aliquot was centrifuged, then was left standing for 60 minutes and examined for fructose; K5: the aliquot was centrifuged, left for 120 minutes and then the supernatant was taken and examined for fructose; K6: the aliquot was allowed to stand for 30 minutes, then centrifuged, and the supernatant was examined for fructose content; K7: the aliquot was allowed to stand for 60 minutes, then centrifuged, and supernatant examined for fructose content; K8: the aliquot after standing for 120 minutes was centrifuged and supernatant examined for fructose content. Fructose was examined using a Semi-Automated Method.

The procedure of Fructose Test with Semi-Automated Method

Using the BTS-350 semiautomatic machine, a colorimetric procedure followed an enzymatic process involving phosphoglucoisomerase and hexokinase enzymes.
A spectrophotometer was used to detect NADPH. To measure fructose with the BTS 350 semiautomatic device, the reagent mixture Reagent A (PIPES 70 mmol/l, NADP+ 1.2 mmol/l, hexokinase > 15 U/ml, phosphoglucose isomerase > 10 U/ml, preservative, pH 7), Reagent B (ATP > 15 mmol/l, glucose-6-phosphate dehydrogenase > 10 U/ml, preservative, pH 9), and fructose standard (D-fructose 75 mg/dL equivalent to 375 mg/dL or 28 mmol/l fructose according to the sample dilution factor) were used. Samples were added to reagents and put in a unique tube made of Teflon to be sucked automatically by the machine until, within a few minutes, the value of fructose content will appear. After that, the fructose concentration needs to be calculated manually.

Statistical analysis

The data obtained were analyzed statistically with the Graph Pad Prism 10 software package. Data normality test was conducted with the Shapiro Wilk test, and comparative analysis between variables was tested with the Wilcoxon Sign Rank Test for non-normally distributed data, and paired t-test for normally distributed data.

Results

The results of this study were obtained from samples of 13 subjects divided into eight aliquots. The general characteristics of the study participants and observational data of macroscopic analysis of ejaculate are shown in Table 1. In this study, the results of ejaculate ejaculate of infertile men with azoospermia criteria were 8%, severe oligozoospermia 23%, oligoteratozoospermia 23%, asthnezoospermia 31%, and teratozoospermia 15%.
Table 2 shows no significant difference between fructose levels measured in the aliquot that was immediately examined after centrifugation compared to fructose levels measured in aliquots that were left standing after centrifugation for 30 ($p = 0.100$) and 60 ($p = 0.133$) minutes. However, there was a significant difference between the level measured immediately after centrifugation compared to the level measured in the aliquot left for 120 minutes ($p = 0.036$). These results suggest that 60 minutes may be the recommended most extended time limit for measurement of fructose after centrifugation, as after that time, fructose levels may drop not resembling real levels.

Table 1.
The general characteristics of the study participants and observational data of macroscopic analysis of ejaculate.

General characteristics of participants and results of macroscopic ejaculate analysis	Mean ± SD	Median (min-max)
Age (years old)	32.69 ± 4.76	-
Abstinence (day)	-	4 (2-7)
Volume (ml)	3.39 ± 1.31	-
Liquefaction (minute)	-	-
pH	-	7.5 (7-8,1)
Viscosity (cm)	-	< 2

Table 2.
Comparison of fructose concentrations (Mean ± SD) in seminal plasma standing for 0, 30, 60, or 120 minutes after centrifugation of ejaculate.

Aliquot	N	Fructose (mmol/L)
0 minutes	13	15.77 ± 4.81
30 minutes	13	15.18 ± 5.34 [a]
60 minutes	13	14.87 ± 5.47 [b]
120 minutes	13	14.23 ± 5.76 [c]

[a] $p = 0.100$; [b] $p = 0.133$; [c] $p = 0.036$.

In the comparison between fructose levels that are left standing before centrifugation, there was a significant difference between immediate measurement and measurements aliquots that are left standing for 60 minutes ($p = 0.012$) and 120 minutes ($p < 0.001$) before centrifugation. The decrease in fructose levels between direct examination and 120 minutes was highly significant. This finding indicates that leaving samples for more than 60 minutes before centrifugation significantly decreases the measurement of the level of fructose content (Table 3).
The results of the measurements of fructose in the different eight aliquots were plotted on a graph, showing that aliquots that were left standing for a period of time and

Table 3.
Comparison of fructose concentrations (Mean ± SD) in seminal plasma obtained from centrifuged ejaculate after standing for 0, 30, 60, or 120 minutes.

Aliquot	N	Fructose (mmol/L)
0 minutes	13	15.77 ± 4.81
30 minutes	13	15.30 ± 5.64 [a]
60 minutes	13	14.25 ± 5.84 [b]
120 minutes	13	10.51 ± 7.21 [c]

[a] $p = 0.294$; [b] $p = 0.012$; [c] $p = < 0.001$.

due to different centrifugation speeds and the number of samples measured. The present study used a centrifugation speed of 4000 rpm for 30 minutes, while the research conducted by Lu et al. used a centrifugation speed of 3000 x g for 15 minutes. Although research conducted by Lu et al. showed that different centrifugation speeds have little effect on fructose levels, remaining spermatozoa or non-cellular components (7, 8), including zinc, alpha-glucosidase, citric acid may mildly affect the fructose level (9).
The difference may also be due to differences in sample size being the sample size in the present study was 13

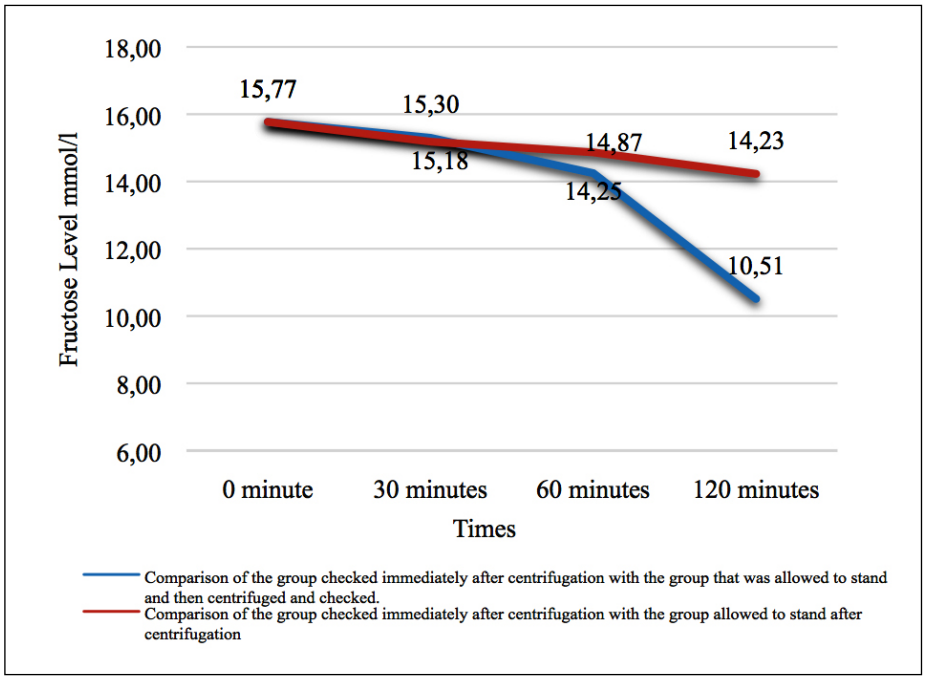

Figure 1.
Comparison of mean fructose levels measured in aliquots immediately centrifugated and then allowed to stand compared to levels measured in aliquots allowed to stand before centrifugation.

then centrifuged showed a more significant decrease in fructose levels than measurement in aliquots that were centrifuged and then left standing for a period of time. Our findings suggest that the best procedure for fructose examination is immediate centrifugation with measurement within 60 minutes. Levels of fructose in samples left standing before centrifugation tend to be lower than real levels (Figure 1).

Discussion

The results showed that there was a significant difference in fructose levels in the aliquots that were immediately centrifuged after liquefaction and then immediately checked compared to the aliquots that, after centrifugation, were allowed to stand for 120 minutes before measurement ($p = 0.036$). There is a significant difference in fructose levels in the aliquots immediately checked compared to those allowed to stand 60 minutes and 120 minutes after liquefaction, then centrifuged and checked ($p = 0.012$ and $p < 0.001$).
The difference in results in the present study for the aliquots that were allowed to stand 120 minutes after centrifugation compared to the results of *Lu et al.* may be people, while the study research by Lu et al. included 20 subjects.
The data from the aliquots examined directly after centrifugation compared to those examined after a standing period before centrifugation showed no significant difference after a 30-minute standing (15.78 mmol/L and 15.30 mmol/L), but after 60- and 120-minute standing the fructose levels decreased significantly (14.25 mmol/L and 10.51 mmol/L). The finding of no difference of fructose level after 30 minutes standing align with the research of *Elzanaty* and *Malm* although they found no significant difference between ejaculate fructose levels examined also 60 minutes, and 90 minutes after ejaculation (10).
The fructose levels of the aliquots that were allowed to stand for 60 and 120 minutes before centrifugation were significantly lower than those allowed to stand for only 30 minutes. This is in line with research conducted by *Lu et al.*, although the method used is different. Lu et al. used the resorcinol method, while this study used the enzymatic method. They reported a difference in the fructose content of ejaculate that was allowed to stand for 2 hours compared to those directly examined, and fructose levels significantly lower after 4 hours with

respect to 0 or 2 hours. In other words, the fructose concentration decreased with the length of standing time (7). The results in this study are also in line with the research conducted by *Andrade-Rocha*, in which fructose concentration was significantly lower in the samples examined after 120 minutes of liquefaction (11).
The process of fructolysis causes a decrease in fructose levels in ejaculate. This process is influenced by many factors such as concentration, motility and metabolism of spermatozoa, temperature, pH, and other substances in the ejaculate (9). An increase in spermatozoa concentration requires more fructose, which decreases fructose levels in ejaculate and vice versa (12). Increased fructose levels due to decreased fructose utilization can be caused by reduced spermatozoa, abnormal spermatozoa morphology, and decreased spermatozoa activity. Low fructose levels can be caused by good spermatozoa motility (13). There is a positive correlation between the motility rate of spermatozoa and the fructolysis rate in human ejaculation (9). Very low temperatures can stop all spermatozoa metabolic activity, affecting fructose utilization and resulting in a slow decline in fructose levels (14).

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Conflict of interest: The authors declare no potential conflict of interest.