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Stability of Free β-hCG in the Routine Screening of Down Syndrome in the First Trimester of Pregnancy

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Abstract: Stability of free β-human chorionic gonadotropin in maternal blood upon storage at ambient and refrigerator temperatures as well as stability under conditions simulating postal transport were studied.

A group of 26 women in the first trimester of pregnancy were included in the study, blood samples were taken during the routine check-up. Blood samples were aliquoted stored in different conditions that should mimic the transportation and then analysed for free β-hCG.

No significant differences were found in the free β-hCG levels in samples after 24 and 48 hours resp., stored in refrigerator (2–8 °C) without separation. In samples stored in laboratory temperature without separation the average concentration increased from 11 to 20%. Six blood samples were stored at 35 °C for 5 hours and then in laboratory temperature. In this group the average increase of results was from 14.3% to 132.2%. Separation of the sera for Down syndrome screening in 4 hours after withdrawal is necessary. Cooling during any storage, including transportation is highly recommended as the preanalytical phase has a high impact for the analysis.

Introduction

Human chorionic gonadotropin (hCG) free β-subunit measurement, together with pregnancy associated protein A (PAPP-A) is used as a screening test for Down syndrome during the first trimester of pregnancy. Combination of both parameters has higher distinctive value; however it also brings some problems. Difficulties arise mainly from low stability of free β-hCG subunit. In collected blood, free β-subunit does not dissociate but it is subjected to nicking and other forms of degradation even in properly separated serum. Nicked free β-hCG lacks peptide linkages between either β-subunit residues 44 and 45 or β-subunit residues 47 and 48. The percentage of nicked free β-hCG increases after the second month of pregnancy [1, 2]. Nicked free β-hCG is less stable, dissociates readily [3] and probably may have different stability upon transportation [4]. Comparing time dependent dissociation of free β-hCG in sera of pregnant and non-pregnant women, dissociation of free β-hCG is much slower in the group of non-pregnant patients. This fact brought about the hypothesis that pregnancy-associated factors accelerate the rate of dissociation [5, 6]. For comparative studies, it is therefore important to study samples collected from pregnant women at the gestational age generally used for Down syndrome screening (10 to 12 weeks).

Several papers have described the stability of free β-hCG subunit measurements in sera samples [4, 7, 8, 9]. None, however, has dealt with the issue of storage conditions during postal sample transportation and its influence to the analytical results. In Czech Republic, postal transportation is still widely used due to organization of prenatal screening where big centres are supplied by several distant points within the region. To decide whether transportation conditions have an impact on the results of measurement, study mimicking those storage conditions

has to be carried out. We present a study of stability of hCG and its effect on free b-subunit levels in serum samples at ambient and refrigerator temperatures with the time of storage 1–3 days. This period is a usual time range of delivery to the place of analysis. Another aim of our experiment was to detect the possible interference of the temperature fluctuation during the standard shipping conditions without cooling.

Material and Methods

Experimental design

Experimental group included 26 women in the first trimester of pregnancy undergoing first trimester prenatal screening in the Institute of Clinical Biochemistry and Laboratory Diagnostics of Charles University in Prague. Women were recruited for the study randomly during one week; all of them signed an informed consent with the study. Five blood samples were collected in vacutainers from each of them. The first one was processed in accordance with the recommendations for good laboratory practice valid in our laboratory (http://ukb.lf1.cuni.cz/index.php), so the blood was separated by centrifugation in 10 min after temperature reduction and formation of the clot. Then, serum was removed and free β-hCG was determined within 4 hours. These values were considered a reference.

Half of the samples (group 2 and 4) were stored in refrigerator and others were left in conditions of room temperature (group 3 and 5) 24 or 48 hours before the concentration of free β-hCG in the samples was analysed. To mimic transport conditions, six samples (group 6 and 7) were placed into the thermostat immediately after collection, stored for 5 hours at 35 °C and than left in room temperature. Table 1 summarises storage and separation conditions for all the samples.

Assay

Free β-hCG was assayed on BRAHMS KRYPTOR. This system uses the TRACE (Time Resolved Amplified Cryptate Emission) technology based on a non-radiative transfer of energy. This transfer takes place between two fluorescent tracers: a donor, europium cryptate, and an acceptor, XL665, (a phycobilisome obtained from red algae). In immunometric assay both are bound to an antibody.

TRACE is method for first trimester screening of Down Syndrome which is recommended by Foetal Medicine Foundation, London UK.

Statistical analysis

Statistical analysis was performed using Statistica 7.1 CZ software (StatSoft, Praha 6, Czech Republic). All values are reported in mean±standard deviation or as shift percentages. The paired t-test for dependent samples was used to analyze the within-group variation. Differences were considered to be statistically significant at the 0.001 level (2-tailed).

Results

Samples were divided into seven groups. In the first one (fresh serum samples) was the free β-hCG level 85.05 ± 44,66 mg/l. These values served as a reference for following calculations.

Material of the second and fourth groups was stored for 24 resp. 48 hours in 2–8 °C before analyses. No significant differences in the free β-hCG levels were found in samples after 24 and 48 hours resp., stored in refrigerator without separation, obtained values being 85.47 ± 45.10 μg/l, resp. 94.18 ± 48.32 μg/l.

Material of groups 3 and 5 was stored without separation for 24 resp. 48 hours in 20–22 °C before analyses, the average concentration of free β-hCG level was 87.23 ± 42.38 μg/l, and 102.8 ± 45.9 μg/l resp. This increasing is statistically significant at the 0.001 level

Samples of groups 6 and 7 which were incubated 5 hours after drawing at 35 °C, were stored for 19 resp. 43 hours in 20–22 °C before analyses. In average, 75.12 ± 51.16 μg/l, resp. 156.9 ± 71.8 μg/l of free β-hCG were obtained. Figure 1 shows increasing concentration of free β-hCG in groups 6 and 7 which were incubated 5 hours at 35 °C. Such conditions should mimic thermal conditions during transportation. These two groups contain only 6 samples and as it was found that levels of free β-hCG were rising significantly, the subsequent testing was omitted for economical reasons. Also these groups were not accepted for statistical processing; only graphic presentation was used.

The rise of free β-hCG expressed in percentiles is evident. While the increase in samples of group 2 and 4, stored in refrigerator was small (0.48 resp. 0.13%),

Table 1 – Experimental scheme – different storage condition for each group

Group	Sampling	after 24 hours	after 48 hours
1 reference	separation of serum, analysis up to 4 hours after sample collection		
2	stored at 2–8 °C	separation of serum, analysis of free β-hCG	
3	stored at 22 °C	separation of serum, analysis of free β-hCG	
4	stored at 2–8 °C	stored at 2–8 °C	separation of serum, analysis of free β-hCG
5	stored at 22 °C	stored at 22 °C	separation of serum, analysis of free β-hCG
6	stored at 22 °C + 5 hours at 35 °C	separation of serum, analysis of free β-hCG	
7	stored at 22 °C + 5 hours at 35 °C	stored at 22 °C	separation of serum, analysis of free β-hCG

the average increase in group 3 and 5 was significant (11.9% and 20.2% respectively). In blood samples placed after sampling in 35 °C for 5 hours and stored 19 resp. 43 hours in laboratory temperature conditions, the free β-hCG level increased in average for 14.3% resp. 132.2%. Increase of the free β-hCG level was highly significant and distinct especially in samples with longer time of storage. All groups were tested for normality before applying t-test. Except for the group 6 all groups fitted into normal distribution. Small number of samples in groups 6 and 7 not allowed statistical analysis; nevertheless the increase of free β-hCG levels was evident.

Results of paired tests of differences for all groups are in Table 2. No significant changes in free β-hCG concentrations were observed in group 2 and 4. Significant changes were observed in groups 3 and 5. The correlation between groups 3 and 4 was near to limit of significance.

Levels of PAPP-A were not significantly changed in any condition described below. Evaluation of risk with higher level of free β-hCG has been calculated. In these concrete cases the final risk was increased 5 to 12 times in samples exposed to higher temperature (group 6 and 7). In samples stored in room temperatures the risk increased 1.2–2.0 times. For other samples the change of risk is not significant.

Our study of free β-hCG concentration changes caused by time and temperature of storage confirms that free β-hCG can be adversely affected during transportation. Especially higher temperature may result to higher concentration of this marker and may increase the false positive rate of prenatal screening.

Discussion

Prenatal screening frequently requires sample transportation. Different storage conditions during preanalytical phase can cause alterations of results due to the limited stability of the estimated parameter. Literature provides several studies on

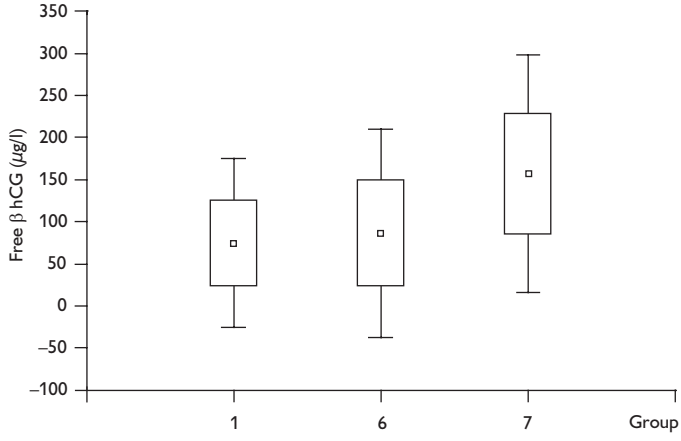

Figure 1 – Free β-hCG in groups 6 and 7 after 5 hours incubation in 35 °C versus reference group 1

the stability of the first trimester prenatal screening markers performed on both separated and unseparated blood [1, 8, 10]. Most of these studies were aimed at free β-hCG only due to its possible release during degradation of intact hCG [13]. In study [11] significant changes in the free β-hCG levels were found. Measurements after 70 hours of storage in 22 °C for separated serum and even after 34 hours for whole blood sample were done in order to simulate adverse conditions during transportation. However, it has been reported that the dissociation of hCG with time is highly dependent on temperature and is much slower in sera from non-pregnant women giving rise to the hypothesis that pregnancy-associated factors accelerate the rate of dissociation [5, 6].

The study of stability of maternal blood markers for Down syndrome screening consisted of five freeze-thaw cycles. Main increase of free β-hCG levels was 5.5% on average which had only a limited impact on the screening performance [12]. Contrary to that, comparison of stability of maternal serum markers (AFP, uE3, total hCG and free β-hCG) with increasing temperature shows that there must be a significant increase of the measured results for samples withdrawn in summer that were sent from external blood drawing sites [15]. Previous studies have used temperatures between 20–22 °C. Considering real conditions especially in continental climate with both summer and winter extremes, mild conditions (20–22 °C) occurred in fact very rarely. Sancken and Bahner [4] found that free β-hCG levels increased 100% after incubation of whole blood at 30 °C for 72 hours, but they observed only an increase about 20% at 20°C. In other study, for samples separated before storage, increase of results about 10% was found and they expect even higher levels in non-separated blood [7, 9].

Kardana and Cole further assumed that hCG nicking activity comes from microbes, and that free β-subunit levels can be stabilized for shipping and longer storage by antibiotic/antimycotic additives [1]

Table 2 – Paired Differences of free β-hCG levels for all group with different storage conditions

Group	Mean free β-hCG	Standard deviation	Degree of freedom	Significance (2-tailed)
1–2	–0.427	2.152	25	0.321464
1–3	0.945	4.000	19	0.303947
1–4	–9.131	6.959	25	**0.000001**
1–5	–14.645	6.116	19	**0.000000**
2–3	1.585	3.545	19	0.060082
2–4	–8.704	6.940	25	**0.000001**
2–5	–14.005	6.418	19	**0.000000**
3–4	–9.335	4.113	19	**0.000000**
3–5	–15.590	6.085	19	**0.000000**
4–5	-6.255	3.301	19	**0.000000**

Changes in bold cells are significant at the $p < 0.001$ level (2-tailed).

Improper storage during preanalytical phase affects substantially results of the tests and may increase the false positive rate of prenatal screening programs for aneuploidies, temperature being more critical factor than delay in delivery [14].

The large study published in 2006 [16] concluded that the levels of first- and second-trimester Down syndrome screening markers can be measured reliably if the sample is centrifuged, refrigerated before shipment and analyzed in the laboratory up to one week after withdrawal. A conservative approach would be to avoid testing later than 6 days after taking the sample, with the intent to keep shipping delays to a minimum.

In accordance to the previous literature, we observed significantly higher levels of free β-hCG in specimens that were submitted to higher environmental temperatures and to processing delays. This would result in an increase of the false positive rate of screening programs, increasing costs and unnecessary stress for the women. Because this is a controllable source of variation, it should be minimized.

Conclusion

Our study of stability of free b-hCG in real conditions of collection and transport of blood samples showed that results were reliable, when samples of free β-hCG were separated immediately (max. 4 hours after drawing), stored at 2–8°C and analyzed within 3 days. Higher temperature during transport affected level of free β-hCG significantly more than time of the storage. However, it is responsibility of the laboratory to ensure reliable results and therefore control convenient transport conditions.

Use of the standard sample collection and transportation protocols can avoid artificial changes in free β-hCG, and only in such conditions clinical utilization of this analyse in screening for Down syndrome can be fully accomplished. On the other side, participation of free β-hCG is relatively small in evaluation of the risk. It is worth considering if this parameter should be even determined in case we cannot secure enough preanalytic care for the sample.

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