has been demonstrated in cytotrophoblasts obtained from the placental beds of preeclamptic pregnancies *(14)*. Increased incidence of apoptosis involving syncytiotrophoblasts has also been reported in preeclampsia *(15)*. Further research on the correlation between fetal DNA concentrations and incidence of placental apoptosis may help to confirm this link.

The potential clinical implication of our findings is that maternal plasma fetal DNA might be used as marker for predicting preeclampsia. However, our data showed that there was overlap in the fetal DNA concentrations between the preeclamptic and control groups. This implies that a relatively low sensitivity and specificity would result if maternal plasma fetal DNA measurement is used as the sole predictor for preeclampsia. Thus, ROC curve analysis (MedCalc 5.0) revealed that the best discrimination between the preeclamptic and control groups was obtained at a fetal DNA concentration of 33.5 genome-equivalents/mL. The sensitivity and specificity at this cutoff concentration were 67% (95% confidence interval, 41–87%) and 82% (95% confidence interval, 65–93%), respectively. The area under the ROC curve was 0.778 (SE = 0.073; 95% confidence interval, 0.639–0.882). Nonetheless, our data open up the possibility of predicting preeclampsia using maternal plasma fetal DNA, especially when used with other biochemical markers such as corticotropin-releasing hormone, α-fetoprotein, inhibin A, and activin A *(16, 17)*. In this regard, it is also important to explore the use of fetal DNA markers outside the Y chromosome so that this type of analysis can be extended to pregnant women carrying female fetuses. Assays that are potentially applicable in this capacity have recently been described *(18–20)*. Finally, our preliminary data would serve to stimulate further large-scale studies to explore the possible correlation of this new marker to the severity of the disease.

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Effects of Repeated Freeze-Thaw Cycles on Concentrations of Cholesterol, Micronutrients, and Hormones in Human Plasma and Serum, *George W. Comstock,[1*] Alyce E. Burke,[1] Edward P. Norkus,[2] Gary B. Gordon,[3] Sandra C. Hoffman,[1] and Kathy J. Helzlsouer[1]* ([1] Department of Epidemiology, Johns Hopkins School of Public Health, Hagerstown, MD 21742; [2] Department of Medical Research, Our Lady of Mercy Medical Center, Bronx, NY 10470; [3] Searle, Skokie, IL 60077; * address correspondence to this author at: Johns Hopkins Training Center for Public Health Research, 1302 Pennsylvania Ave., Hagerstown, MD 21742-3197; fax 301-797-3669, e-mail gcomstock@mindspring.com)

The effects of repeated freeze-thaw cycles on concentrations of various analytes in plasma or serum were of little interest until the growth of plasma and serum banks during the latter part of the 20th century. By 1996, the number of such banks used primarily for cancer research had grown to 115 *(1)*. Many also exist for other purposes, such as the WHO Serum Reference Banks *(2)* and banks associated with cardiovascular studies such as the Multiple Risk Factor Intervention Trial (MRFIT) and the Atherosclerosis Risk in Communities (ARIC) Study *(3, 4)*. Although the need for repeated freezing and thawing of samples can be minimized by storing banked specimens in several small containers *(5)*, it often is necessary to use plasma or serum that has already undergone one or more freeze-thaw cycles. When this occurs, reviewers of re-

search protocols or manuscripts may question the validity of data obtained from these specimens.

The scientific literature provides few answers to such questions. Medline contains no appropriate key word or phrase for searching; comments on the effects of freeze-thaw cycles often are limited to a few sentences in publications that focus on stability during long-term storage or on assay methodology. The present study was designed to add information to this sparse literature.

In 1991, 10 healthy adult volunteers, 5 men and 5 women, each donated 120 mL of blood. The purpose of the study was explained to each person along with the risks and lack of individual benefit. Approval of the study was granted by the Committee on Human Volunteers of the Johns Hopkins School of Hygiene and Public Health before study initiation.

Blood was collected into three 20-mL Vacutainer Tubes containing 286 USP units of sodium heparin (cat. no. 6406; Becton Dickinson) to obtain plasma, and into four 15-mL plain Vacutainer Tubes to obtain serum. The three plasma samples and four serum samples were pooled for each donor. Each of these 10 plasma and serum donor pools was divided into 7 primary aliquots, 2 for baseline assays after one freeze-thaw cycle, and 1 each for the assays after 2, 3, 4, 6, and 10 freeze-thaw cycles. From each primary plasma aliquot, five secondary aliquots were prepared for assays of ascorbic acid, cholesterol, dehydroepiandrosterone and dehydroepiandrosterone sulfate, other hormones, and micronutrients. Each primary serum aliquot was similarly divided except that no ascorbic acid assays were done on serum. Plasma and serum samples were kept in sterile flasks set in ice-water except during the actual fluid transfers. For the ascorbic acid assays, 0.5 mL of freshly prepared 100 g/L metaphosphoric acid was added to 0.5 mL of plasma before freezing; the mixture was then immediately frozen at −70 °C. All aliquoting and thawing were done under dim yellow light.

Secondary aliquots for freeze-thaw cycle 2 were removed from the freezer, thawed once in cold water, allowed to stand at room temperature for 30 min, and refrozen at −70 °C. These samples were not thawed again until the time of assay, thereby completing the second freeze-thaw cycle. Samples for freeze-thaw cycle 3 were thawed as above, refrozen at −70 °C, thawed as above again, and then kept frozen until thawed for assay. This system of thawing and refreezing was repeated so that freeze-thaw cycle 4 had its fourth freeze-thaw cycle completed at the time of assay. These procedures were continued for freeze-thaw cycles 6 and 10.

All specimens remained frozen in insulated containers with dry ice during shipment to the assay laboratories. All specimens were assayed in the random order assigned before shipment. This procedure ensured that the order of assaying was random with respect to both the donor of the specimens and to the freeze-thaw cycle.

Ascorbic acid assays were performed with 2,4-dinitrophenylhydrazine as chromogen *(6)*. Other micronutrients (retinol, total carotenoids, α-carotene, β-carotene, cryptoxanthin, lycopene, lutein, α-tocopherol, and γ-tocopherol) were assayed by reversed-phase HPLC *(7)*. Cholesterol concentrations were determined enzymatically *(8)*. Assays for dehydroepiandrosterone and its sulfate were performed by RIA (Wien Laboratories, Succasunna, NJ) *(9)*. The procedure was that suggested by the manufacturer except that dehydroepiandrosterone was extracted with a 1:1 mixture (by volume) of dichloromethane and hexane.

For males, the hormones for assay were estrone, estradiol, testosterone, and sex hormone-binding globulin (SHBG); for females, androstenedione, follicle-stimulating hormone, luteinizing hormone, progesterone, and SHBG were determined. In male serum, estrone and estradiol were measured by RIA after extraction and Celite chromatography *(10)*. Testosterone was measured by RIA using a method from DPC. SHBG was measured using an immunoradiometric method from Orion. In female serum, androsterone and progesterone were measured by RIA using methods from ICN.

The results for duplicate aliquots that had undergone only one freeze-thaw cycle were used to estimate imprecision (as CVs) for the individual assays. The mean value of the two concentrations for each analyte was used as the cycle 1 value. For each analyte, the linear regression equation ($y = a + bx$) for mean analyte concentrations (y) on the number (x) of freeze-thaw cycles was calculated. The average amount of change per cycle (b) was divided by the estimated value before any freezing (a), and the result was expressed as a percentage of the calculated prefreezing value.

The imprecision (CV) for plasma and serum was similar. For cholesterol and the micronutrients, the median CV was 7.4%, with a range of 1.2% (serum cholesterol) to 19% (plasma α-carotene). For hormones, the variability was greater. The median CV was 14% and the range was 2.5% (androstenedione in serum) to 58% (progesterone in serum). For seven analytes, CVs were >15%: progesterone in plasma and serum (41% and 58%, respectively); estradiol in plasma and serum (17% and 39%); estrone in plasma and serum (27% and 22%); and dehydroepiandrosterone in plasma (17%).

The mean change associated with each freeze-thaw cycle (and its accompanying 30-min exposure to room temperature) was <4% of the estimated prefreeze concentration for all analytes and <2% for nearly all of them (Table 1). Five analytes had changes per cycle of 2–4%. All of these five were hormones: estrone in plasma and serum; estradiol in serum; and SHBG and dehydroepiandrosterone sulfate in plasma. In general, there was a slight tendency for concentrations to decrease with each successive freeze-thaw cycle and for these changes to be least for the first three cycles. Analyte concentrations in serum were somewhat less likely to be affected by freezing and thawing than concentrations in plasma.

In this study, three cycles of freezing and thawing had almost no effect on concentrations of cholesterol, micronutrients, and most of the hormones investigated (data not shown). For estrone, estradiol, testosterone, and SHBG, there was appreciable variation within the first

three cycles, but the degree of variability was considerably less than the respective CVs. Although there were greater losses after 6 or 10 cycles for a few other analytes, these were too small to have a meaningful effect on results.

With one exception, other studies agree with these findings. With respect to cholesterol, serum specimens from 10 baboons were subjected to 10 freeze-thaw cycles *(11)*. The results indicated "a stable serum cholesterol during repeated freezing-thawing".

In a report on assay methodology, Driskell et al. *(12)* stated that "Vitamins A and E in serum were found to be stable to freezing and thawing (seventeen freezing and thawing cycles over a period of five weeks)". Brioch et al. *(13)* concluded that eight freezing and thawing cycles of hypercarotenemic serum made "no significant differences in the levels of carotenoids or retinyl palmitate". In a study on the stability of vitamin E, Gunter et al. *(14)* reported that two samples stored at −70 °C for 3 months had losses of 13% and 21% after the fifth cycle. Regression analysis of data reported by Nierenberg *(15)* showed that after seven freeze-thaw cycles of a single specimen, plasma concentrations of β-carotene decreased by 0.3% of the estimated baseline value per cycle. A more comprehensive study by Hsing et al. *(16)* used 15 aliquots of pooled sera. After four freeze-thaw cycles, no changes in the concentrations of retinol, total carotenoids, β-carotene, lycopene, or total tocopherols were >0.3% of the estimated prefreezing value.

Hsing et al. *(16)* also looked at changes in serum concentrations of several hormones after three freeze-thaw cycles of five pooled specimens. The estimated change per cycle was 0.7% for testosterone, 1.4% for luteinizing hormone, and 3% for follicle-stimulating hormone. Wickings and Nieschlag *(17)* assayed four aliquots of plasma after 33 freeze-thaw cycles. They concluded that "Repeated freezing and thawing of plasma samples does not affect the plasma concentrations of T (testosterone) and Adione (androstenedione)".

On the basis of previous reports and the results of this study, we believe that repeated freezing to −70 °C and thawing has no meaningful effects on the plasma and serum concentrations of a considerable number of micronutrients and hormones.

Table 1. Mean change in concentrations of selected analytes per freeze-thaw cycle expressed as a percentage of the estimated initial value.

	Change, %			
	Plasma		Serum	
Analyte	Mean	SD	Mean	SD
Cholesterol	−0.38	0.23	−0.01	0.11
Micronutrients				
Retinol	−0.16	0.35	−0.32	0.17
Total carotenoids	−1.34	0.37	−0.28	0.23
α-Carotene	−0.63	0.44	0.12	0.50
β-Carotene	−0.84	0.64	0.15	0.63
Cryptoxanthin	−0.97	0.53	−0.48	0.47
Lutein	−0.75	0.49	−0.70	0.46
Lycopene	−1.54	1.08	−0.13	0.48
Ascorbic acid	0.14	0.12		
α-Tocopherol	−1.36	0.41	−0.44	0.09
γ-Tocopherol	−1.09	0.22	−0.47	0.27
Hormones, female				
Androstenedione	0.34	1.49	−1.09	1.12
FSH[a]	−0.70	0.50	−0.12	0.59
Luteinizing hormone	−1.60	0.87	−0.12	0.34
Progesterone	0.09	2.37	−0.31	5.93
SHBG	0.03	1.28	−0.67	2.37
Hormones, male				
Estrone	−2.21	1.04	−2.23	0.56
Estradiol	−0.33	4.45	−2.45	2.74
Testosterone	1.77	1.62	1.01	2.91
SHBG	2.73	2.65	−1.01	2.10
Hormones, both sexes				
DHEA	−0.19	1.94	−0.14	0.51
DHEAS	3.26	1.36	0.48	1.01

[a] FSH, follicle-stimulating hormone; DHEA, dehydroepiandrosterone; DHEAS, dehydrepiandrosterone sulfate.

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