# Centralized Blood Processing for the Selenium and Vitamin E Cancer Prevention Trial: Effects of Delayed Processing on Carotenoids, Tocopherols, Insulin-Like Growth Factor-I, Insulin-Like Growth Factor Binding Protein 3, Steroid Hormones, and Lymphocyte Viability

**Alan R. Kristal,[1] Irena B. King,[2] Demetrius Albanes,[7] Michael N. Pollak,[4] Frank Z. Stanzyk,[6] Regina M. Santella,[5] and Ashraful Hoque[3]**

[1]Cancer Prevention Program, Division of Public Health Sciences and [2]Public Health Sciences Laboratories, Fred Hutchinson Cancer Research Center, Seattle, Washington; [3]Department of Clinical Cancer Prevention, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; [4]Cancer Prevention Research Unit, Department of Oncology, Jewish General Hospital and McGill University, Montreal, Quebec, Canada; [5]Mailman School of Public Health, Columbia University, New York, New York; [6]Departments of Obstetrics and Gynecology, and Preventive Medicine, University of Southern California Keck School of Medicine, Los Angeles, California; and [7]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland

## Abstract

**This experiment examined the effects of delays in separation and freezing of whole blood components on analytes of interest in studies of prostate cancer prevention, in order to evaluate the feasibility of centralized processing of blood for the multisite Selenium and Vitamin E Cancer Prevention Trial. Blood from 40 healthy men was subjected to four treatment protocols, allowing the contrast of immediate processing to delays of 32, 72, and 144 hours. At 32 hours, simulating refrigerated storage and overnight shipping, there was a 2.9% decrease (95% confidence interval, 0.7-5.1) in insulin-like growth factor-I (IGF-I) but no significant change in carotenoids, tocopherols, testosterone, 3α-androstanediol glucuronide (AAG), sex hormone–binding globulin (SHBG) or insulin-like growth factor binding protein 3 (IGFBP3). A 144-hour processing delay, simulating weekend blood collection or shipping delay, resulted in significant changes in γ-tocopherol (−1.5%), IGF-I (−5.7%), IGFBP3 (−2.9%), SHBG (−4.0%), testosterone (+4.7%), and AAG (+5.5%). The rank-order and intraclass correlations between analytes from blood processed immediately and those subjected to delayed processing were 0.96 or higher for carotenoids, tocopherols, AAG, and SHBG, and between 0.87 and 0.95 for IGF-I, IGFBP3, and testosterone. A 32-hour delay decreased lymphocyte viability from 82.5% to 75.0% ($P = 0.45$), but a 72-hour delay decreased viability to 36.8% ($P < 0.001$). Overnight shipping and centralized processing is an acceptable approach to blood collection in large multisite trials examining the cancer-related measures proposed in the Selenium and Vitamin E Cancer Prevention Trial. Longer processing delays, however, have small but statistically significant effects on many analytes and substantially decrease lymphocyte viability.**

## Introduction

Collection and storage of blood samples in large, multicenter studies can be very expensive and procedurally complex, especially if blood samples require laboratory processing before shipment to a central storage facility. One approach to simplifying blood collection is to ship unprocessed blood to a central facility, where separation of blood components, aliquotting, labeling, and storage are done by trained personnel using carefully standardized materials and methods. However, some blood analytes may degrade during the period between collection and arrival at a central processing facility, which could introduce error and bias into subsequent analyses. As part of the planning for the Selenium and Vitamin E Cancer Prevention Trial (1), we evaluated whether the proposed blood collection protocol, based on refrigerated storage and overnight shipping, as well as several scenarios that would delay receipt of blood samples at a central facility, such as weekend blood collection and weather-related shipping delays, would affect analytes of interest in studies of prostate cancer prevention. This manuscript gives results of an experiment simulating effects of blood storage and shipping, examining tocopherols, carotenoids, testosterone, insulin-like growth factor I (IGF-I), insulin-like growth factor binding protein 3 (IGFBP3), and viability of lymphocytes for immortalization. Several small studies have evaluated the effects of delayed blood processing on some of these analytes; none have examined the range of scenarios likely to be encountered in large, multisite studies. If delayed processing has little effect on plasma analytes, it would allow substantial savings in blood processing costs in multisite studies requiring blood collection and storage.

## Materials and Methods

**Blood Processing.** Fasting blood samples from 40 healthy male volunteers, ages 22 to 50, were collected into four 7-mL royal-topped tubes with EDTA as an anticoagulant. Bloods were subjected to four treatments simulating likely field

conditions (Table 1), which consisted of several combinations of refrigerated storage (4°C) and shipping (storage in a 1.5-inch-thick styrene foam Bio-Mailer with −1°C gel-packs, using a Styrofoam divider to isolate tubes from gel-packs to prevent freezing). Shipping container temperatures were 10°C at 24 hours, 14°C at 48 hours, and 21°C at 72 hours. After treatment, blood samples were centrifuged and plasma was stored at −70°C. Buffy coats were washed twice with red cell lysing solution. Leukocytes were then resuspended in RPMI 1640 (Sigma-Aldrich), centrifuged, and finally resuspended in freeze media containing RPMI 1640 supplemented with fetal bovine serum, streptomycin, penicillin, and 15% filtered DMSO. Leukocytes were subject to rate-control freezing before final storage in liquid nitrogen. All procedures were completed under sterile conditions.

**Laboratory Assays.** Tocopherols and carotenoids were assayed using reverse-phase high-performance liquid chromatography (2, 3). IGF-I and IGFBP3 were assayed by ELISA with reagents from Diagnostic Systems Laboratory (Webster, TX) as described previously (4). Testosterone was measured by RIA after organic solvent extraction and Celite column partition chromatograph (5). 3α-Androstanediol glucuronide (AAG) was quantified by direct RIA (6) and sex hormone–binding globulin (SHBG) was measured by direct chemiluminescent immunoassay using the Immulite analyzer (Diagnostic Products Corporation, Inglewood, CA). For all assays, samples for an individual were analyzed in the same batch, and intraassay coefficients of variation were as follows: α-carotene (3.0%), β-carotene (2.6%), lutein + zeaxanthin (2.4%), β-cryptoxanthin (1.6%), lycopene (3.0%), α-tocopherol (1.4%), γ-tocopherol (2.2%), IGF-I (1.4%), IGFBP3 (2.0%), AAG (4.5%), testosterone (9.5%), and SHBG (4.1%). To immortalize lymphocytes, cells were thawed and the concentration was adjusted to 3 million cells/mL in RPMI 1640 supplemented with 40% fetal bovine serum, penicillin and streptomycin, and 2 mmol L-glutamine. One milliliter was plated on 24-well plates, and 12 μL of cyclosporin at 1 mg/mL and 1 mL of EBV supernatant (B95.8) were added to each well (grown in Iscove's media with 10% FCS). Plates were incubated at 37°C for 10 days. One milliliter of old media was replaced with 1 mL of fresh RPMI 1640 media after colonies were visualized. If there was no visible growth of colonies by 21 days, 1 mL of old media was replaced with 0.5 mL RPMI 1640 media and 0.5 mL virus stock. Samples were followed for up to 30 days before final determination of lymphocyte viability.

**Statistical Methods.** Effects of blood treatments were described as (*a*) the arithmetic mean of paired differences between treatment A and all other treatments, and (*b*) the percentage change from treatment A, calculated as $e^{\Delta}$, where $\Delta$ is the mean of paired differences in log-transformed values. Paired *t* tests were used to determine statistical significance. Spearman rank-order correlations and intraclass correlations were used to examine agreement between treatment A and all other treatments. The intraclass correlation excluded the mean difference between the two measures, using the formula described in Armstrong et al. (ref. 7, p. 104), which corresponds to an analyses that would include a covariate indicating the length of processing delay.

**Table 1. Blood processing treatments, simulating variable storage and shipping times**

| Treatment | Description | Time between collection and freezing (h) |
|---|---|---|
| A | Immediate processing and storage | <2 |
| B | Optimal study protocol<br>8 h refrigerated storage<br>24 h shipping with gel pack | 32 |
| C | Minor delay, due to evening or weekend clinic<br>24 h refrigerated storage<br>48 h shipping with gel pack | 72 |
| D | Major delay, due to weather emergency or accidental mishandling<br>72 h refrigerated storage<br>72 h shipping with gel pack | 144 |

## Results

Table 2 gives mean values of each analyte from blood processed immediately after collection (treatment A), as well as mean and percentage differences due to simulated delays in processing (treatments B, C, and D). Delayed processing had very modest effects on carotenoids and tocopherols; nevertheless, there were small decreases for β-cryptoxanthin that reached statistical significance for percentage but not mean change and small decreases in both percentage and mean change in γ-tocopherol. IGF-I decreased incrementally with longer storage time, reaching a −5.75% decrease ($P < 0.001$) for treatment D. IGFBP3 also decreased significantly with longer storage time, although the effect was only −2.9% for treatment D. There were no effects of delayed processing on testosterone, AAG, and SHBG except for treatment D. After the long processing delay simulated in treatment D, testosterone and AAG increased by ~5% and SHBG decreased by 4%.

All rank-order correlations between measures from blood processed immediately and measures from blood subjected to delayed processing were high (Table 3). Both rank-order and intraclass correlations were 0.96 and higher for carotenoids, tocopherols, AAG, and SHBG, with no suggestion of decreasing correlation with longer delays before processing. Correlations were somewhat lower for IGF-I, IGFBP3, and testosterone, ranging from 0.87 to 0.95.

Delayed processing profoundly affected lymphocyte viability. Transformation was successful for 33 out of 40 samples (82.5%) from treatment A; 15 out of 20 samples (75%) for treatment B (versus treatment A, $P = 0.45$), and only 7 out of 19 (36.8%) samples from treatment C (versus treatment A, $P < 0.001$). Given the poor results from treatment C, treatment D was not evaluated.

## Discussion

This study evaluating the feasibility of shipping unprocessed blood samples to a central processing facility found no significant effects of delays of up to 32 hours (overnight shipping) on blood analytes, with the exception of a small decrease in IGF-I. Longer processing delays, which would be expected with weekend blood collection or shipping delay, resulted in significant decreases in γ-tocopherol, IGF-I, IGFBP3, and SHBG, and increases in testosterone and AAG. There was a small but significant decrease in percentage but not mean change in β-cryptoxanthin with a delay of 144 hours, which due to this inconsistency, was not considered meaningful. However, even with a processing delay of 144 hours, no analyte concentration was affected by >6%. Furthermore, for most analytes, the rank-order and intraclass correlations between blood samples processed immediately and those subjected to delayed processing were >0.95, and in no cases did correlations drop below 0.87. In contrast to results on blood analytes, lymphocyte viability was modestly decreased after simulated overnight shipping and next day processing, but fell by more than half after a 72-hour delay.

There are few studies that have systematically examined the effects of shipping unprocessed blood on analytes of interest in

**Table 2. Effects of delayed processing on serum concentrations of carotenoids, tocopherols, IGF-I, IGFBP3, AAG, testosterone, and SHBG ($n$ = 40)**

| Analyte | Treatment* | | | |
|---|---|---|---|---|
| | A<br>$\bar{X} \pm SD$† | B<br>$\bar{\Delta} \pm SE$‡,<br>$\Delta$% (95% CI)§ | C<br>$\bar{\Delta} \pm SE$‡,<br>$\Delta$% (95% CI)§ | D<br>$\bar{\Delta} \pm SE$‡,<br>$\Delta$% (95% CI)§ |
| α-Carotene (μg/dL) | 6.4 ± 4.8 | 0.11 ± 0.09,<br>−0.1 (−2.2 to 2.1) | 0.16 ± 0.13,<br>−0.0 (−2.8 to 2.8) | 0.16 ± 0.11,<br>0.3 (−2.5 to 3.3) |
| β-Carotene (μg/dL) | 28.7 ± 24.4 | 0.49 ± 0.27,<br>0.8 (−0.3 to 1.9) | 1.36 ± 0.86,<br>1.9 (−1.0 to 4.9) | 0.76 ± 0.46,<br>2.2 (−0.3 to 4.7) |
| Lutein + Zeaxanthin (μg/dL) | 21.4 ± 9.8 | −0.08 ± 0.09,<br>0.7 (−0.0 to 1.5) | 0.08 ± 0.27,<br>−0.2 (−1.5 to 1.2) | 0.05 ± 0.09,<br>−0.0 (−0.8 to 0.8) |
| β-Cryptoxanthin (μg/dL) | 12.2 ± 9.8 | −0.22 ± 0.20,<br>−2.8 (−4.3 to 1.2) | 0.10 ± 0.26,<br>−1.6 (−3.6 to 0.4) | −0.30 ± 0.20,<br>3.2 (−5.0 to −1.3) |
| Lycopene μg/dL | 39.9 ± 12.5 | −0.24 ± 0.26,<br>−0.6 (−1.9 to 0.8) | 0.60 ± 0.80,<br>0.9 (−1.8 to 3.7) | −0.78 ± 0.77,<br>1.3 (−1.4 to 4.1) |
| α-Tocopherol (μg/dL) | 1,222 ± 542 | −3.4 ± 5.6,<br>0.2 (−0.1 to 0.3) | 19.1 ± 12.4,<br>1.0 (−0.1 to 0.3) | −4.6 ± 6.5,<br>−0.3 (−1.24 to 0.7) |
| γ-Tocopherol (μg/dL) | 133 ± 72.7 | −1.5 ± 0.09,<br>−1.1 (−2.3 to 0.0) | −1.4 ± 0.07‖,<br>−0.7 (−1.7 to 3.2) | −2.1 ± 0.09‖,<br>−1.5 (−2.6 to −0.3) |
| IGF-I (ng/mL) | 206 ± 54.9¶ | −10.7 ± 2.4**,<br>−2.9 (−5.1 to −0.7) | −14.7 ± 3.2**,<br>−3.6 (−6.4 to −0.5) | −16.8 ± 2.7**,<br>5.7 (−7.7 to −3.3) |
| IGFBP3 (ng/mL) | 3,033 ± 589¶ | −28.3 ± 40.5,<br>−1.3 (−3.9 to 1.4) | 2.0 ± 49.3,<br>0.1 (−3.2 to 4.2) | −91.3 ± 42.0‖,<br>2.9 (−5.2 to 0.5) |
| AAG (ng/mL) | 6.6 ± 3.3 | 0.2 ± 0.1,<br>1.5 (−0.8 to 3.9) | −0.0 ± 0.1,<br>−1.2 (−4.5 to 2.1) | 0.5 ± 0.2††,<br>5.5 (2.4 to 8.7) |
| Testosterone (ng/dL) | 630 ± 236¶ | 5.1 ± 13.5,<br>0.1 (−3.5 to 3.7) | 9.1 ± 14.7,<br>1.4 (−2.1 to 5.1) | 25.7 ± 15.7,<br>4.7 (0.3 to 9.5) |
| SHBG (nmol/L) | 39.4 ± 22.5¶ | 0.4 ± 0.5,<br>1.3 (−1.8 to 4.6) | −0.2 ± 0.6,<br>−0.2 (−3.5 to 3.1) | −1.3 ± 0.5††,<br>−4.0 (−6.5 to −1.2) |

*See Table 1 for descriptions of treatments.
† Geometric mean ± SD, except where noted.
‡ Mean change from treatment A ± SE.
§Mean percentage change from treatment A and 95% confidence interval.
‖$P < 0.05$.
¶Arithmetic mean ± SD.
**$P < 0.001$.
††$P < 0.01$.

cancer epidemiology. Kley and Rick (8) examined the effects of storing whole blood for 4 days on steroid hormones in six samples, and found a 5% decrease in testosterone and 5% increase in SHBG (both $P < 0.05$). Craft et al. (9) examined effects of storing plasma for 24 hours on carotenoids and tocopherols in four samples, and found a nonsignificant 12% decrease in α-tocopherol and much smaller changes in carotenoids. Hankinson et al. (10) examined the effects of a 28-hour processing delay on carotenoids and tocopherols in 12 samples, and found a 5.5%/day decrease in β-carotene and a 0.9%/day decrease in α-tocopherol (both not significant), with much smaller effects on other carotenoids and γ-tocopherol. The same study also examined the effects of a 48-hour delay on steroid hormones in samples from 10 women, and found increases of 9.5%/day in testosterone and 1.3%/day in SHBG (both not significant). Key et al. (11) reported the effects of a 24-hour processing delay on carotenoids and steroid hormones in 28 samples, and found decreases of 8.7% ($P < 0.05$) for α-carotene and 6.7% (not significant) for lycopene, and nonsignificant increases in testosterone of 3.2% for females and 1.8% for males. Hankinson et al. (4) reported an intraclass correlation of 0.98 for IGF-I and 0.96 for IGFBP3 among an unstated number of samples processed immediately and delayed for 24 and 36 hours. Most of these studies were based on very small samples and were underpowered to detect moderate effects of delayed processing. In general, however, these studies found no or very modest effects of delayed processing.

**Table 3. Spearman rank-order and intraclass correlations comparing carotenoids, tocopherols, IGF-I, IGFBP3, AAG, testosterone, and SHBG ($n$ = 40)**

| Analyte | Treatments* | | | | | |
|---|---|---|---|---|---|---|
| | A versus B | | A versus C | | A versus D | |
| | S† | I‡ | S | I | S | I |
| α-Carotene | 0.99 | 0.99 | 0.98 | 0.99 | 0.98 | 0.99 |
| β-Carotene | 0.99 | 0.99 | 0.98 | 0.99 | 0.99 | 0.99 |
| Lutein + Zeaxanthin | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| β-Cryptoxanthin | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| Lycopene | 0.98 | 0.99 | 0.97 | 0.96 | 0.96 | 0.96 |
| α-Tocopherol | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 |
| γ-Tocopherol | 0.99 | 0.99 | 0.98 | 0.99 | 0.99 | 0.99 |
| IGF-I | 0.95 | 0.96 | 0.93 | 0.94 | 0.94 | 0.95 |
| IGFBP3 | 0.89 | 0.90 | 0.88 | 0.87 | 0.91 | 0.91 |
| AAG | 0.98 | 0.99 | 0.97 | 0.98 | 0.97 | 0.98 |
| Testosterone | 0.95 | 0.94 | 0.96 | 0.93 | 0.93 | 0.92 |
| SHBG | 0.98 | 0.99 | 0.98 | 0.99 | 0.98 | 0.99 |

NOTE: Log-transformed, with the exceptions of IGF-I, IGFBP3, testosterone, and SHBG.
*See Table 1 for description of treatments.
†Spearman rank-order correlation.
‡Intraclass correlation.

Several nonexperimental studies have examined the effects of delayed processing on lymphocyte viability. A single study reported a profound drop in cell viability after processing delays of 3 or more days, which is consistent with our findings (12). Others report successful transformation after delays of up to 72 hours (13, 14) and up to 6 days (15), however, none of these present supporting data. None of these previous studies is comparable to the experiment reported here because all used acid citrate dextrose as an anticoagulant. Acid citrate dextrose is optimal for cell viability, but it is not the preferred

anticoagulant when blood samples will be used for multiple purposes. Overall, these results suggest that in studies requiring viable lymphocytes, delay of blood processing beyond 32 hours without using specialized collection methods such as acid citrate dextrose tubes is inappropriate.

We conclude that overnight shipping and centralized processing is an acceptable approach to blood collection in large multisite trials examining the cancer-related measures proposed in the Selenium and Vitamin E Cancer Prevention Trial. Longer processing delays, however, have small but significant effects on IGF-I, IGFBP3, and testosterone, and in studies for which precise measures of these analytes are critical, investigators may wish to obtain repeat blood draws to replace samples subjected to delays longer than 3 days. Finally, lymphocyte viability is satisfactory from blood samples after overnight shipping, but investigators planning to immortalize lymphocytes will need repeat blood draws to replace samples delayed for longer than 32 hours.

## References


1. Klein EA, Thompson IM, Lippman SM, et al. SELECT: the Selenium and Vitamin E Cancer Prevention Trial rationale and design. Prostate Cancer Prostatic Dis 2000;3:145–51.
2. Zhang C, Williams MA, King IB, et al. Dietary intake and plasma vitamin C status in relation to preeclampsia risk. Epidemiology 2002;13:409–16.
3. Kalman DA, Goodman GE, Omenn GS, et al. Micronutrient assay for cancer prevention clinical trials: serum retinol, retinyl palmitate, α-carotene, and β-carotene with the use of high-performance liquid chromatography. J Natl Cancer Inst 1987;79:975–82.
4. Hankinson SE, Willett WC, Colditz GA, et al. Circulating concentrations of insulin-like growth factor I and risk of breast cancer. Lancet 1998;351:1393–6.
5. Goebelsmann U, Bernstein GS, Gale JA, et al. Serum gonadotropin, testosterone, estradiol and estrone levels prior to and following bilateral vasectomy. In: Lepow IH, Crozier R, editors. Vasectomy: immunologic and pathophysiologic effects in animals and man. New York: Academic Press; 1979.
6. Narang R, Rao JN, Savjani G, et al. Radioimmunoassay kit for the quantitative measurement of androstanediol glucuronide in unextracted serum. 17th National Meeting of the Clinical Ligand Assay Society Poster #12, Chicago (IL): 1991.
7. Armstrong BK, White E, Saracci R. Principles of exposure measurement in epidemiology. Oxford: Oxford University Press; 1992.
8. Kley HK, Rick W. Influence of storage and temperature on steroid analysis in plasma and blood. J Clin Chem Clin Biochem 1984;22:371–8.
9. Craft NE, Brown ED, Smith JC. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem 1988;34:44–8.
10. Hankinson SE, London SJ, Chute CG, et al. Effect of transport conditions on the stability of biochemical markers in blood. Clin Chem 1989;35: 2313–6.
11. Key T, Oakes S, Davey G, et al. Stability of vitamins A, C, and E, carotenoids, lipids, and testosterone in whole blood stored at 4°C for 6 and 24 hours before separation of serum and plasma. Cancer Epidemiol Biomarkers Prev 1996;5:811–4.
12. Reidy JA, Wheeler VA. Sample age and Epstein-Barr virus transformation of cryopreserved lymphocytes. In Vitro Cell Dev Biol Anim 1992;28A:383–4.
13. Louie LG, King M-C. A novel approach to establish permanent lymphoblastoid cell lines: Epstein-Barr virus transformation of cryopreserved lymphocytes. Am J Hum Genet 1991;48:637–8.
14. Pressman S, Rotter JI. Epstein-Barr virus transformation of cryopreserved lymphocytes: prolonged experience with technique. Am J Hum Genet 1991;49:467.
15. Beck JC, Beiswanger CM, John EM, et al. Successful transformation of cryopreserved lymphocytes: a resource for epidemiological studies. Cancer Epidemiol Biomarkers Prev 2001;10:551–4.