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Reproducibility of Erythrocyte Polyamine Measurements and Correlation With Plasma Micronutrients in an Antioxidant Vitamin Intervention Study

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Abstract Erythrocyte polyamine measurements have been previously investigated as candidate biomarkers for hyperproliferation and recently as a potential intermediate endpoint in clinical chemoprevention trials with difluoromethylornithine, an inhibitor of polyamine biosynthesis. This study was performed to determine the reproducibility of erythrocyte polyamine measurements and their possible correlation with plasma micronutrients in seven healthy adults in an antioxidant vitamin intervention study. As part of this cross-over intervention study, three subjects took β-carotene (31.4 mg/day) plus D-α-tocopherol acetate (720 IU/day) supplements during the first 3 months and four subjects took the supplements during the second 3 months. Heparinized blood samples were collected at baseline and every month over total 6 months for simultaneous determination of erythrocyte polyamines and plasma micronutrients by the high-performance liquid chromatographic method. For all the measures of erythrocyte polyamines the intraindividual variation was smaller than that between subjects, and three or four measurements required to accurately characterize long-term erythrocyte polyamines for an individual. The intra-class correlations were moderately high for all erythrocyte polyamine measurements, indicating a good reproducibility for intra-individual erythrocyte polyamine measurements. Based on monthly values, significant inverse correlations were found between erythrocyte spermidine and the plasma levels of retinol ($r = -0.50$) and lutein ($r = -0.52$). There were also significant inverse associations between erythrocyte spermine and plasma levels of α-tocopherol ($r = -0.29$), lutein ($r = -0.44$), lycopene ($r = -0.29$), β-cryptoxanthin ($r = -0.30$), and total carotenoids ($r = -0.29$). The effects of supplementation upon the associations between erythrocyte polyamines and plasma nutrient levels were additionally addressed. The results indicate an acceptable longitudinal reproducibility of erythrocyte polyamine measurements, support the hypothesis that erythrocyte polyamine measurements may be correlated with plasma levels of certain nutrients, and suggest a further biomarker application in cancer prevention trials involving dietary modifications or specific relevant micronutrients.

Key words: polyamines, erythrocyte, biomarker, reproducibility, plasma micronutrients, antioxidant, intervention, cancer prevention

INTRODUCTION

Polyamines are small, aliphatic, highly charged organic cations, which play an important role in cellular proliferation and differentiation [Raina and Janne, 1975; Tabor and Tabor, 1984; Pegg and McCann, 1982]. Spermidine (Spd), spermine (Spm), and their precursor putrescine (Put) are the principal polyamines found within virtually all mammalian cells and tissues. The majority of circulating polyamines are transported by red blood cells (RBC) that contain more than 95% of Spd and Spm in the blood [Cohen et al., 1976; Moulinoux et al., 1984, 1991]. The erythrocyte polyamine (EP) measurements have been clinically applied as a biochemical index of cellular proliferation in antiproliferative cancer therapy [Cipolla et al., 1993, 1994; Quemener et al., 1986; Bergeron et al., 1989;Chatel et al., 1987; Moulinoux et al., 1988] and recently as a potential biomarker endpoint in a clinical chemoprevention trial with an inhibitor of polyamine

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biosynthesis [Pendyala et al., 1993]. However, a heterogeneous distribution of polyamines has been noted in anatomically confined colonic epithelial [Higuchi and Wang, 1995]. A number of technical and biological factors have been also reported to affect the measurement of polyamine contents [Hixson et al., 1994]. These observations lend further request to measure the longitudinal reproducibility prior to use EP measurements as an intermediate endpoint in cancer prevention trials.

There is extensive epidemiological evidence suggesting a protective role for plasma antioxidant micronutrients such as carotenoids and tocopherols in cancer risk. This possible protection against cancer by micronutrients has been indicated to involve inhibited cellular proliferation [Schwartz et al., 1990, 1993; Ramanathan et al., 1994; Schwartz and Shklar, 1992]. Since EP measurements may reflect cellular proliferation, it can therefore be hypothesized that increased plasma micronutrient status, and more successful antiproliferation will correspond with decreased EP measurements. The EP measurements may be thus co-modulated by certain dietary micronutrients.

This present study was performed in seven healthy individuals by an antioxidant vitamin intervention study. Estimates of the reproducibility of EP measurements and the correlations between EPs and plasma micronutrients were investigated. The potential influence of β-carotene plus α-tocopherol supplementation upon EP measurements was subsequently addressed. The number of samples required to characterize a usual EP level of an individual, and the potential utility of EPs as biomarker in dietary intervention and/or prevention trials was additionally discussed.

MATERIALS AND METHODS

Chemicals and Reagents

The capsules of β-carotene labeled at 15 mg/pill and D-α-tocopherol acetate labeled at 400 IU/pill were donated by Henkel Corporation (La Grange, IL). The purity of the capsules measured by our established high-performance liquid chromatographic method (HPLC) [Franke et al., 1993] is 104.7% for β-carotene and 90% for D-α-tocopherol acetate, respectively. That is, the exact contents of the β-carotene and D-α-tocopherol acetate capsules are 15.7 mg/pill and 360 IU/pill, respectively. In addition, we observed that the β-carotene capsule contains about 6.4% of α-carotene (1.0 mg/pill of α-carotene). Therefore, the results reported herein have been adjusted based on the purity. All other chemicals and chromatographic standards, unless indicated otherwise, were purchased from Sigma Chemical Company (St. Louis, MO).

Subjects and Study Design

Seven healthy, nonsmoking volunteers (five females and two males, median age of 52 years and a range from 35 to 69 years) were entered this single-blind study between August 1993 and January 1994. Details of these subjects are given in Table 1. After base-line values were determined, three subjects received 15.7 mg of β-carotene and 360 IU of D-α-tocopherol acetate twice daily (with breakfast and dinner) for the first 3 months. The other four subjects took the supplements during the second 3 months as part of this cross-over design. The heparinized blood samples were collected at baseline and every month for 6 months from all subjects after overnight fast. RBC and plasma were separated

TABLE I. Characteristics of the Study Subjects and Erythrocyte Polyamine Measurements During the β-Carotene Plus α-Tocopherol Supplemental and the Non-Supplemental Periods

Subjects			Spd (nmol/ml RBC)[a]		Spm (nmol/ml RBC)[a]	
No.	Sex	Age (yr)	Non-supplemental	Supplemental	Non-supplemental	Supplemental
1	F	53	15.99 ± 1.84	11.30 ± 1.44	6.49 ± 0.43	5.23 ± 0.78
2	M	70	20.33 ± 2.47	18.30 ± 3.24	7.91 ± 1.35	7.33 ± 0.55
3	F	45	28.34 ± 4.59	27.57 ± 6.20	8.58 ± 0.99	8.75 ± 1.52
4	M	42	17.74 ± 1.31	14.50 ± 4.41	8.82 ± 0.86	7.42 ± 0.98
5	F	36	11.72 ± 1.99	9.60 ± 1.30	8.91 ± 1.30	8.80 ± 1.64
6	F	61	19.81 ± 3.45	18.40 ± 3.38	10.29 ± 1.53	9.67 ± 1.71
7	F	40	24.60 ± 3.50	20.33 ± 5.04	12.10 ± 0.51	12.22 ± 0.51

[a]Mean ± SD using all assays for the supplemental period (n = 3) and non-supplemental period (n = 4).

by centrifugation. RBC samples were immediately transferred to laboratory for polyamine analysis, and plasma samples were stored at −70°C until analysis for micronutrients. This study was approved by the Committee on Human Subjects of the University of Hawaii.

Polyamine Analysis

Polyamines were measured in the RBC extracts in triplicate or duplicate using a reverse-phase gradient HPLC with fluorescence detection of the dansylated polyamines by modification of our previously published method [Higuchi and Wang, 1995].

RBC (100 μl) were osmotically lysed into 510 μl of distilled water after washing (3 x) with ice-cold phosphate buffered saline (PBS). After the addition of 1,12-diaminododecane as an internal standard (final concentration, 3 nmol/ml), proteins were precipitated in the final 5% of perchloric acid. Protein-free supernatants (500 μl) were vortexed with saturated sodium carbonate (350 μl) and 1% dansyl chloride in acetone (400 μl), then the mixtures were incubated at 60°C for 1 h. Dansylated polyamines were extracted in toluene, dried, then redissolved in acetonitrile (100 μl) just prior to HPLC analysis.

A reverse-phase chromatography procedure using a Perkin-Elmer (Norwalk, CT) Pecosphere-3 × 3 CR C_{18}, 33 × 4.6 mm I.D. cartridge column (3 μm particle size) with an heptanesulfonate (10 mM, pH 3.4) to acetonitrile gradient at a flow rate of 2.5 ml/min was used, with fluorescence detection set for excitation at 330 nm and emission at 470 nm. Peak areas were calculated using a Dynamax MacIntegrator (Rainin Instrument Co., Inc., Emeryville, CA).

Using this method, the limit of detection is ~ 0.05 nmol/ml RBC for Put, Spd, Spm, and their acetyl-derivatives. The coefficient of variations (CV) for the measurement of EPs in triplicate or duplicate samples were always less than 2%. The determinations of day-to-day precision and accuracy of measurements for the polyamines were carried out based on eight standard curves generated on 8 different days, and the coefficients of variation of the ratios of slope to intercept in the 8 standard curves were less than 4%.

Micronutrient Analysis

Extraction and analysis of plasma carotenoids and tocopherols were done by HPLC as described in our previously published method [Franke et al., 1993].

Briefly, plasma proteins were precipitated with ethanol containing butylated-hydroxy-toluene (BHT) as antioxidant and three internal standards followed by repeated (3 x) hexane extraction of the lipophilic micronutrients. The combined hexane layers were dried under nitrogen and redissolved in the HPLC mobile phase consisting of methanol:dichloromethane:acetonitrile (65:25:10), BHT (0.025%) as antioxidant, and aqueous bis-tris-propane (2 ml/L of 0.5 M, pH 7.0) as buffer. Twelve carotenoids, retinol, γ-, and α-tocopherol were separated on a Spherex 5-μm C18 column (250 × 4.6 mm) (Phenomenex, Torrance, CA) and monitored by a dual multiple wavelength detector at each individual compound's absorption maximum. Levels were determined using peak areas and calibration curves of authentic standards.

Although 12 carotenoid fractions were used to calculate total plasma carotenoids, only the five major peaks (α-carotene, β-carotene, lycopene, lutein, and β-cryptoxanthin) are reported in detail here. Plasma ascorbic acid was measured with the dichlorophenolindophenol method [Vanderjagt et al., 1986]. Total plasma cholesterol was measured enzymatically in a cholesterol oxidase/peroxidase system using a diagnostic's kit from Sigma Chemical Co. (St. Louis, MO).

Analytical accuracy and reliability were verified by participation in the National Institute of Standards and Technology "round robin" for micronutrient analysis with results consistently within 6% of the mean values reported for all "core" laboratories and CV not greater than 4%, with the exception of α-carotene for which the CV was 6.8%.

Statistical Analysis

In the analysis of the data, all the EP and some micronutrient measures were log transformed, as log (x + 1), in order for the distributions to approximate normality. The monthly values for EPs during vitamin supplement and non-supplement periods were modeled with a one-way random effect ANOVA [Snedecor and Cochran, 1989] to obtain estimates of intra- and inter-individual variance.

The estimated number of repeat measurements necessary to rank individuals correctly to their long-term analyte levels was computed

using the formula proposed by McAvay et al. [McAvay et al., 1988]:

$$\left(\frac{E_\beta}{1-E_\beta}\right)\frac{S_w^2}{S_B^2}.$$

Where E_β is error term for the regression coefficient between the measured and the true underlying values for an individual, and s_W^2 and s_B^2 are the observed within and between subject variances computed by ANOVA. A value of E_β = 0.90 was chosen because the observed regression coefficient would be kept no more than 10% different from the true regression coefficient. The extent of within-subject reproducibility was assessed by the intra-class correlation (ICC). ICC was computed using the formula as follows [Snedecor and Cochran, 1989]:

$$ICC = \frac{S_W^2}{S_B^2 + S_W^2}.$$

In essence, ICC quantifies the extent of overall agreement between the repeated monthly measurements either in the supplemental period or in the non-supplemental period. Power to detect differences in variables is increased if the ICC is high. If ICC were equal to one (maximum values), then there would necessarily be exact agreement between the repeat measurements for all the subjects.

Linear regression was used to model respectively the effect of vitamin intervention on EP measurements and plasma micronutrients. Correlations between EPs and micronutrient levels were computed by Pearson's correlation coefficients (r values), using the monthly values for each subject in the supplemental (n = 21 observations), non-supplemental (n = 27), and total experimental period (n = 48), respectively.

RESULTS

Polyamine Analysis

Figure 1 shows the typical polyamine chromatograms for both standards and RBC sample. A set of seven commercially purchased standards was used to determine retention times of the individual polyamines. Following the sample extract and HPLC procedure described in Materials and Methods, Spd and Spm were detectable but not Put in the RBC extracts of all the subjects over the 6-month experimental period. The Spd levels were always higher than the Spm

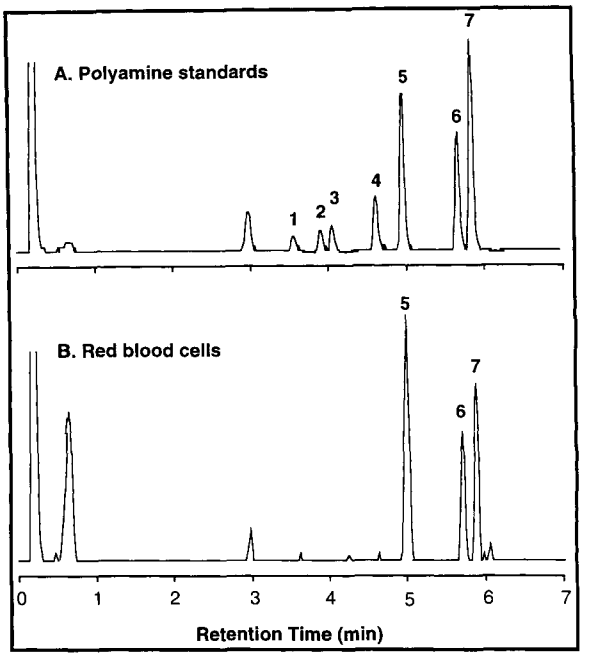

Fig. 1. Typical polyamine chromatograms obtained with standards (**A**) and red blood cells (**B**). *1*, acetylspermidine; *2*, cadaverine; *3*, putrescine; *4*, acetylspermine; *5*, spermidine; *6*, diaminododecane (internal standard); *7*, spermine. Red blood cells were osmotically lysed into water, and erythrocyte polyamines were extracted by perchloric acid. The protein-free supernatants of erythrocyte polyamine extracts were dansylated and re-extracted prior to HPLC analysis. A reverse-phase chromatography procedure using a Perkin-Elmer (Norwalk, CT) Peco-sphere-3 × 3 CR C_{18}, 33 × 4.6 mm I.D. cartridge column with an heptanesulfonate (10 mM, pH 3.4) to acetonitrile gradient at a flow rate of 2.5 ml/min was used. Dansylated polyamines were detected using fluorescence detection set for excitation at 330 nm and emission at 470 nm, and the peak areas were used to calculate the values of erythrocyte polyamine measurements.

levels, and the ratios of Spd to Spm ranged from 1.07 to 3.94. The levels of Spd ranged from 8.30 to 33.80 nmol/ml RBC, and those of Spm ranged from 4.60 to 12.80 nmol/ml RBC. Acetylated polyamines were not detectable in the RBC extracts of all the samples.

Reproducibility of EP Measurements

The characteristics of the subjects and the mean of EP measurements either in the supplemental or non-supplemental periods are listed in Table 1. The sex and age differences were not found due to the small sample size. However, certain intra- and inter-individual variations in the EP measurements were suggested. To visualize the variations suggested in Table 1 for EP

measurements, the variabilities of EP measures were estimated and presented in Table 2.

Table 2 lists the estimated within- and between-subject variations, within- to between-subject variance ratios, and the estimated number of repeat measurements required to characterize an individual with respect to the group. For all analytes intraindividual variation was smaller than that interindividual, and three or four samples appeared to characterize an individual. The reproducibility of EP measurements was additionally assessed by ICC. The ICC ranged from 0.70 to 0.78 and was moderately high in all study groups, indicating a good reproducibility for intra-individual EP measurements in this present study.

Correlations Between EPs and Plasma Micronutrients

Table 3 shows the follow-up changes in mean EP measurements and mean plasma levels of micronutrients for seven subjects. The plasma levels of β-carotene, α-tocopherol, total carotene and α-carotene rose sharply during β-carotene plus α-tocopherol supplementation, while a responsible decrease in plasma γ-tocopherol lev-

TABLE II. The Variabilities of the Erythrocyte Polyamine Measurements in Seven Healthy Individuals Over the Vitamin Supplemental Period (n = 21) and Non-Supplemental Period (n = 28)

Variable	Supplement periods	Within[a]	Between[a]	W/B[b]	No. of measurements required[c]	ICC[d]
Spd	Non-supplemental	0.019	0.069	0.28	2.5	0.78
	Supplemental	0.039	0.100	0.39	3.5	0.72
Spm	Non-supplemental	0.012	0.028	0.43	3.9	0.70
	Supplemental	0.017	0.049	0.35	3.1	0.74

[a]Log-transformed values used in calculations of within and between subject variability.
[b]Ratio of within (W) to between (B) subject variability.
[c]Represents the number of measurements needed to correctly rank at least 90% of the true regression coefficient of individuals as described in Materials and Methods.
[d]Intra-class correlation indicates the extent of within-subject reproducibility of erythrocyte polyamine measurements as described in Materials and Methods.

TABLE III. Changes of Mean Measurements of Erythrocyte Polyamines and Mean Levels of Plasma Micronutrients in Seven Healthy Subjects With β-Carotene Plus α-Tocopherol Supplementation

	Non- supplemental[a]	Supplemental[b]	% Change[c]	P[d]
Polyamines:				
Spd (nmol/ml RBC)	19.79	17.14	−13.4	0.089
Spm (nmol/ml RBC)	9.01	8.49	−5.8	0.392
Micronutrients:				
β-carotene (μg/L)	151	662	+338.4	0.0001
α-carotene (μg/L)	67	133	+98.5	0.0001
Lutein (μg/L)	187	196	+4.8	0.415
Lycopene (μg/L)	201	178	−11.4	0.443
β-cryptoxanthin (μg/L)	85	107	+12.9	0.224
Total carotenoids (μg/l)	982	1,642	+67.2	0.0001
Ascorbic acid (mg/dl)	1.38	1.37	−7.2	0.953
α-tocopherol (μg/L)	10,186	22,962	+125.4	0.0001
δ-tocopherol (μg/L)	491	340	−30.7	0.051
γ-tocopherol (μg/L)	2,211	502	−77.3	0.0001
Retinol (μg/L)	599	592	−1.2	0.830
Cholesterol (mg/dl)	170	165	−2.9	0.728

[a]Mean using all assays for the seven subjects (n = 21).
[b]Mean using all assays for the seven subjects (n = 28).
[c](Supplemental values − non-supplemental values) × 100 ÷ non-supplemental values.
[d]P value from the ANOVA model of log-transformed values, adjusted for month and supplemental group.

els. The EP measurements and the other plasma nutrient levels did not change statistically.

Table 4 lists the correlation coefficients among EPs and plasma micronutrient levels, using the monthly values of the measurements for each subject over the study period. Based on monthly values, statistically significant inverse correlations were observed between erythrocyte spermidine and the plasma levels of retinol ($r = -0.50$) and lutein ($r = -0.52$). There were also significant inverse associations between erythrocyte spermine and plasma levels of α-tocopherol ($r = -0.29$), lutein ($r = -0.44$), lycopene ($r = -0.29$), β-cryptoxanthin ($r = -0.30$), and total carotenoids ($r = -0.29$). There were no significant correlations between EPs and plasma levels of the other nutrients listed. The associations between EPs and plasma nutrient levels were influenced somewhat by supplementation. Although β-carotene was positive associated in both groups, there was no significant correlation when combined together. The retinol correlation was weakened with supplementation, while lutein was inversely associated in both groups. The inverse relationship of cholesterol and Spd was stronger and significant among nonsupplemental period, but the positive correlation of α-carotene and Spd was noted among supplemental period. On the other hand, the inverse association of lutein and Spm was strengthened with supplementation. The lycopene, β-cryptoxanthin, and total carotenoids were not inversely correlated in both groups, while the inverse association between α-tocopherol and Spm was weakened during supplementation.

DISCUSSION

A prerequisite to the use of EP measurements as a measure of risk for hyperproliferation or as an intermediate marker in intervention studies is that EP measurements must have high reliability. The availability of repeat blood samples drawn monthly for 6 months in this antioxidant vitamin intervention study gave us the opportunity to study the reproducibility of EP measurements. The methodological issue of assay precision was addressed in this blinded study by repeated EP measurements using aliquot samples in triplicate or duplicate. The HPLC analysis method, which was based on our prior publication [Higuchi and Wang, 1995], yielded highly reproducible replicate measurements (CV ≤ 2% in the same day and CV ≤ 4% day-to-day). An acceptable degree of assay precision was thus demonstrated.

The critical issue regarding the reliability of biomarker is the biological variation. Biological variation includes interindividual differences and intraindividual variation. Intraindividual variation of EP measurements due to temporal fluctuation of polyamines was presently investigated by independently assaying replicate RBC samples taken from different months. The intra-

TABLE IV. Pearson Correlation Coefficients (*r* Values) Between Plasma Micronutrient Levels and Erythrocyte Polyamines Using All the Observations for Individuals

	Spermidine[a]			Spermine[a]		
Micronutrients	Total (n = 48)	Non-supplemental (n = 27)	Supplemental (n = 21)	Total (n = 48)	Non-supplemental (n = 27)	Supplemental (n = 21)
β-carotene[a]	0.158	0.558[b]	0.753[b]	−0.007	0.267	0.129
α-carotene[a]	−0.166	−0.317	0.532[c]	−0.182	−0.156	−0.079
Lutein	−0.515[b]	−0.549[b]	−0.472[c]	−0.441[b]	−0.323	−0.571[b]
Lycopene[a]	−0.143	−0.357	−0.041	−0.286[c]	−0.287	−0.343
β-cryptoxanthin[a]	−0.205	−0.260	−0.081	−0.296[c]	−0.213	−0.340
Total carotenoids[a]	−0.104	−0.400	0.469[c]	−0.287[c]	−0.351	−0.230
Ascorbic acid[a]	0.206	0.185	0.211	−0.008	−0.067	0.023
α-tocopherol[a]	−0.149	−0.098	0.255	−0.293[c]	−0.397[c]	−0.263
δ-tocopherol	0.230	0.330	−0.018	−0.104	−0.095	−0.232
γ-tocopherol[a]	0.262	0.115	0.137	−0.041	−0.349	−0.367
Retinol	−0.495[b]	−0.643[b]	−0.381	−0.165	−0.068	−0.299
Cholesterol	−0.220	−0.386[c]	−0.103	−0.132	0.015	−0.290

[a]Log-transformed as log (x + 1).
[b]$P \leq 0.01$.
[c]$0.01 < P \leq 0.05$.

individual variation over time for the same individual was noted smaller than the interindividual variation (Table 2). Only three or four measurements were required to accurately characterize long-term EPs for an individual, and a moderately high ICC was observed in all groups. Therefore, an acceptable reproducibility for intra-individual EP measurements was demonstrated in this present study.

The effects of supplementation upon EPs and plasma levels of nutrients were further investigated. Table 3 provides information on the extent to which the mean of EP measurements and the plasma nutrient levels was compared in the seven normal individuals during non-supplemental and supplemental periods. Plasma levels of β-carotene and α-tocopherol were significantly increased during supplementation over non-supplemental period by 338% and 125%, respectively. As a result, total carotenoids were elevated about 1.7-fold during supplemental period. As for the increase of plasma α-carotene levels after taking β-carotene, it should be noted that the capsule of β-carotene used in this study was contaminated with α-carotene as described in Materials and Methods. Moreover, the significant decrease of plasma γ-tocopherol after α-tocopherol supplement may be due to a competitive mechanism between the two forms of vitamin E, which was previously reported by other investigators [Handelman et al., 1985; Behrens and Madere, 1987]. Although the observed mean EP measurements decreased respectively by 13% for Spd and 6% for Spm, there was a non-statistically significant decline in EP levels during 3 months of vitamin supplementation. Given the small sample size and the resulting low statistical power, we could not probably conclude there was a real effect of supplementation upon EP levels in this study.

Since a real effect of supplements on EP levels was not observed, we further estimated the correlations of EPs with plasma levels of all the nutrients listed using the monthly values of the measurements for each subject over the experimental period. Statistically significant inverse correlations were observed between erythrocyte Spd and the plasma levels of retinol and lutein (Table 4). There were also significant inverse associations between erythrocyte Spm and plasma levels of α-tocopherol, lutein, lycopene, β-cryptoxanthin, and total carotenoids.

Although the detailed mechanism is not clear, Spd correlated inversely with the plasma retinol levels are not unexpected. We have previously observed that an associated decrease in cellular polyamine levels after exposure of human colonic epithelial cells to retinol [Higuchi and Wang, 1995], and that could be related to the inhibitory effect of retinol on cellular proliferation [Higuchi and Wang, 1995; Quemener et al., 1994; Pegg, 1988; Lotan, 1994; Sporn and Robert, 1983]. As a result, the reasonable inverse association between Spd and plasma retinol levels may suggest a role for quantitative EP analyses as a biomarker application in chemoprevention trials involving retinol or retinoids. In addition, a number of other micronutrients were found to be correlated (Table 4), possibly suggesting that EP measurements may be related to plasma micronutrient status and may be useful as biomarker in certain micronutrient intervention study. In fact, the mechanism by which the polyamines in RBC are synthesized is unknown. Anucleated cells like RBC do not have a mechanism for the synthesis of polyamines, EPs must either be residual from their nucleated erythropoietic precursors and/or the result of uptake from exogenous sources such as food [Cohen et al., 1976; Moulinoux et al., 1984, 1991]. Therefore, EP levels could be affected by dietary and/or lifestyle factors. Because of this small sample size and our primary interest in investigating the reproducibility of EP measurements and their response to antioxidant vitamin supplementation in the conditions of a large-scale future trial, we did not use a controlled diet in this study.

In summary, we have shown a good reproducibility of EP measurements and the correlations of EPs with plasma levels of various nutrients that reflect the composition of the diet and supplements. All these are necessary bases for subsequent studies to use EP measurements as an intermediate endpoint in cancer prevention studies using diet modification or specific relevant micronutrients.

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