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# Tocopherol Contents of Lipoproteins from Frozen Plasma Separated by Affinity Chromatography

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We investigated whether freezing and storage of plasma altered  $\alpha$ -tocopherol levels of whole plasma or the lipoprotein fractions derived from such plasma. Plasma from 24 men, at each of two collection periods, was frozen at  $-20^{\circ}\text{C}$  for six weeks, then high-density lipoproteins (HDL) were separated from low- plus very low-density lipoproteins (LDL-VLDL) by heparin affinity chromatography. Whole plasma and the lipoprotein fractions were analyzed for  $\alpha$ -tocopherol content and compared to counterparts from fresh plasma. Freezing and storage did not reduce  $\alpha$ -tocopherol levels of plasma or the lipoprotein fractions.  $\alpha$ -Tocopherol values from fresh and frozen plasma were highly correlated for both plasma (period 1,  $r = 0.94$ ; period 2,  $r = 0.93$ ) and the LDL-VLDL fractions (periods 1 and 2,  $r = 0.97$ ). Percent distribution of  $\alpha$ -tocopherol between the two lipoprotein fractions was comparable for lipoproteins derived from fresh and frozen plasma. Under the storage conditions used in this study, plasma can be frozen for at least six weeks prior to lipoprotein fractionation with no detectable detrimental effects on  $\alpha$ -tocopherol content of either plasma or lipoproteins.

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$\alpha$ -Tocopherol is a major lipophilic antioxidant that is transported in plasma by lipoproteins. Low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are the major transport vehicles for  $\alpha$ -tocopherol in human plasma (1-3), and the amount of tocopherol carried by these lipoproteins is strongly influenced by their lipid concentrations (4). The amount of tocopherol carried by very low-density lipoproteins (VLDL) from fasting, normolipemic subjects is a minor portion of the total (1,2).

$\alpha$ -Tocopherol has been measured less frequently on lipoprotein fractions than on whole plasma, but recently there has been considerable interest in structural and biochemical properties of lipoproteins as they relate to lipoprotein tocopherol levels (1,2,5-8). This interest in measuring tocopherol levels of lipoproteins suggests that reliable and convenient methods for determining tocopherol levels of human lipoproteins would be useful. We recently reported that commercially available affinity columns allow quick separation of the two major  $\alpha$ -tocopherol-containing lipoproteins from human plasma (9). Using this separation method in combination with high-performance liquid chromatography (HPLC), analysis of lipoprotein tocopherol can be carried out the day of blood collection with complete recovery of  $\alpha$ -tocopherol in lipoprotein fractions.

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Abbreviations: apoB, apolipoprotein B; HDL, high-density lipoproteins; HPLC, high-performance liquid chromatography; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins.

Yet, practical considerations often require delayed sample analysis bringing into question the suitability and effect of storage conditions. While  $\alpha$ -tocopherol appears to be stable for over a year in frozen plasma (10) or serum (11), little is known about how freezing and storage affect subsequent analysis of tocopherol from lipoprotein fractions. It is generally recommended that plasma be held in the liquid state prior to lipoprotein analysis, and that determinations be carried out promptly. However, if samples are to be held more than a few days, then freezing is recommended, in part, to prevent oxidation of unsaturated lipids (12). If lipoproteins rapidly decompose during freezing and storage of plasma, as generally (13,14) but not universally (15-17) believed, then  $\alpha$ -tocopherol levels of lipoprotein fractions could be greatly reduced. It is also possible that redistribution of tocopherols among lipoproteins occurs during freezing and thawing, thus affecting lipoprotein tocopherol levels without altering tocopherol levels of whole plasma.

The objective of this study was to determine whether freezing and storage of plasma would alter  $\alpha$ -tocopherol contents of lipoproteins and thus compromise results obtained following separation of the lipoproteins by affinity chromatography.

## MATERIALS AND METHODS

Twenty-four subjects, 24 to 57 years of age, were chosen at random from 40 nonsmoking men participating in a controlled diet study (18). For that study, applicants with plasma  $\alpha$ -tocopherol levels of less than  $6 \mu\text{g/mL}$  or greater than  $12 \mu\text{g/mL}$  were not accepted as subjects. Study protocols were approved by the Institutional Review boards of the Georgetown University School of Medicine, Washington, D.C., and the National Cancer Institute, DHHS, Bethesda, Maryland.

During two ten-week periods, men took 15 soft gelatin capsules each day with each capsule providing 1 g of fat and 1 IU of all-*rac*- $\alpha$ -tocopherol (0.74 d- $\alpha$ -tocopherol equivalents) as a preservative. During period 1, fat in the capsules was a blend of stripped lard, hormone-free beef tallow and corn oil (48:40:12, w/w/w). During period 2, the capsules contained a concentrate of refined anchovy oil (ROPUFA<sup>®</sup>, Hoffmann-La Roche, Inc., Nutley, NJ) that provided 6.5 g per day of n-3 fatty acids (eicosapentaenoic plus docosahexaenoic acids). For both periods, fat from the diet, including capsules, provided 40% of energy. The minimum intake of  $\alpha$ -tocopherol from diet and capsules was estimated to be 18 d- $\alpha$ -tocopherol equivalents per day. Menus excluded vitamin E-enriched foods, such as fortified breakfast cereals, and the use of vitamin supplements was not allowed. Plasma cholesterol and triglyceride levels of subjects averaged (mean  $\pm$  SEM)  $173 \pm 17.0$  and  $87 \pm 6.9$  mg/dL, respectively, for period 1 and  $169 \pm 6.7$  and  $60 \pm 5.1$  mg/dL for period 2.

Blood was drawn during the tenth week of each period after an overnight ( $\geq 12$  hr) fast. Blood samples, collected

in vacuum tubes containing potassium EDTA (1.5 mg/mL final concentration), were cooled on ice and plasma promptly separated from red blood cells by low-speed centrifugation (1600 × *g* for 20 min) at 4°C. Plasma samples were protected from light at all stages of processing and analysis.

Plasma lipoproteins were fractionated as described previously (9) using heparin affinity columns (LDL Direct, Isolab Inc., Akron, OH). Whole plasma (50 µL) and eluates (200 µL) containing HDL or LDL plus VLDL (LDL-VLDL) were mixed with 1 to 2 mL of 100% methanol and centrifuged at 1600 × *g* for 10 min. Fifty µL of the methanolic extracts were assayed for α- and γ-tocopherol by HPLC using fluorescence detection (19). Each plasma sample and its corresponding lipoprotein fractions were chromatographed, in duplicate, in the order: plasma, HDL, LDL-VLDL.

Fresh samples were processed and analyzed within 10 hr of collection. Replicate plasma samples were frozen at -20°C immediately after separation from red blood cells. Samples were frozen in 0.5-mL aliquots in tightly sealed plastic vials, but no further precautions were taken against oxidation of stored samples. Following six weeks of storage, samples were thawed at room temperature, then fractionated and analyzed under the same conditions as the fresh counterparts. To assure analytical consistency across time, α-tocopherol standards were validated prior to analysis of each storage-period combination using reference standards of reconstituted serum (National Institute of Standards and Technology, Gaithersburg, MD).

An electroimmunoassay (20) for apolipoprotein B (apoB) was used to determine whether freezing and storage of plasma inhibited the binding of apoB-containing lipoproteins to heparin columns. Column eluates from a subset of eight plasma samples were diluted in electrophoresis buffer to a final concentration of 1:12 (v/v) and, for HDL only, to a final concentration of 1:6 (v/v). Fractions were analyzed, in duplicate, in 1.5% agarose gels containing

1.2% antisera to human apoB, raised in rabbits (Behring Diagnostics, La Jolla, CA). Buffers and conditions for electrophoresis have been described (9).

Data were analyzed using the SAS system (SAS Institute, Cary, NC). Correlation coefficients were determined by the Pearson product-moment method, and the paired *t*-test was used to compare fresh and frozen/stored samples for plasma and lipoprotein tocopherol content (21).

## RESULTS

**Plasma α- and γ-tocopherol levels.** Frozen/stored plasma was not different from fresh plasma when compared for α- and γ-tocopherol contents (Table 1). For period 1, plasma α-tocopherol levels were 10.5 ± 0.5 mg/dL (mean ± SEM) for both fresh and frozen/stored plasma. For period 2, plasma α-tocopherol levels were 9.4 ± 0.4 and 9.5 ± 0.4 µg/mL for fresh and frozen/stored plasma, respectively. Mean levels of γ-tocopherol for fresh and frozen/stored plasma were identical for both period 1 (1.5 ± 0.1 µg/mL) and period 2 (1.4 ± 0.1 µg/mL).

Fresh and frozen/stored plasma samples were strongly correlated (Fig. 1) for both plasma α-tocopherol (period 1: *r* = 0.94, *P* < 0.001; period 2: *r* = 0.93, *P* < 0.001) and plasma α-tocopherol (period 1: *r* = 0.93, *P* < 0.001; period 2: *r* = 0.94, *P* < 0.001).

**α-Tocopherol content of lipoprotein fractions.** α-Tocopherol levels of lipoprotein fractions were not reduced as a function of freezing and storage of plasma (Table 1). However, the period 1 value for LDL-VLDL from fresh plasma was 9% lower than its frozen/stored counterpart (6.1 vs 6.7 µg/mL). This difference was not observed for LDL-VLDL at period 2 or for HDL at either period.

Correlations were strong between LDL-VLDL levels of α-tocopherol from fresh and frozen/stored plasma samples (Fig. 2 *r* = 0.97, *P* < 0.001 for both periods). Correlations were weaker for HDL α-tocopherol levels of fresh and frozen/stored plasma (period 1: *r* = 0.73, *P* < 0.001; period

TABLE 1

Tocopherol Levels in Plasma, LDL-VLDL and HDL. Effect of Freezing Plasma Prior to Lipoprotein Fractionation by Affinity Chromatography<sup>a</sup>

	Fresh	Frozen/ stored <sup>b</sup>	Paired <i>t</i> -test	
			Dif ± SE <sup>c</sup>	<i>P</i> <sup>d</sup>
µg/mL				
Period 1				
α-Tocopherol				
Plasma	10.5 ± 0.53	10.5 ± 0.55	<0.1 ± 0.19	NS
LDL-VLDL	6.1 ± 0.44	6.7 ± 0.49	-0.5 ± 0.13	<0.001
HDL	3.8 ± 0.13	3.7 ± 0.15	0.1 ± 0.11	NS
γ-Tocopherol				
Plasma	1.5 ± 0.12	1.5 ± 0.12	<-0.1 ± 0.05	NS
Period 2				
α-Tocopherol				
Plasma	9.4 ± 0.42	9.5 ± 0.38	-0.1 ± 0.16	NS
LDL-VLDL	6.0 ± 0.38	6.0 ± 0.39	<-0.1 ± 0.10	NS
HDL	3.6 ± 0.12	3.6 ± 0.17	<0.1 ± 0.10	NS
γ-Tocopherol				
Plasma	1.4 ± 0.12	1.4 ± 0.12	<-0.1 ± 0.04	NS

<sup>a</sup>Mean ± SEM (N = 24).

<sup>b</sup>Samples stored at -20°C for six weeks prior to lipoprotein fractionation and tocopherol analysis.

<sup>c</sup>Mean difference and standard error (SE) of the difference.

<sup>d</sup>Probability determined by paired *t*-test, *P* > 0.10 = nonsignificant (NS).

## LIPOPROTEIN TOCOPHEROL FROM FROZEN PLASMA

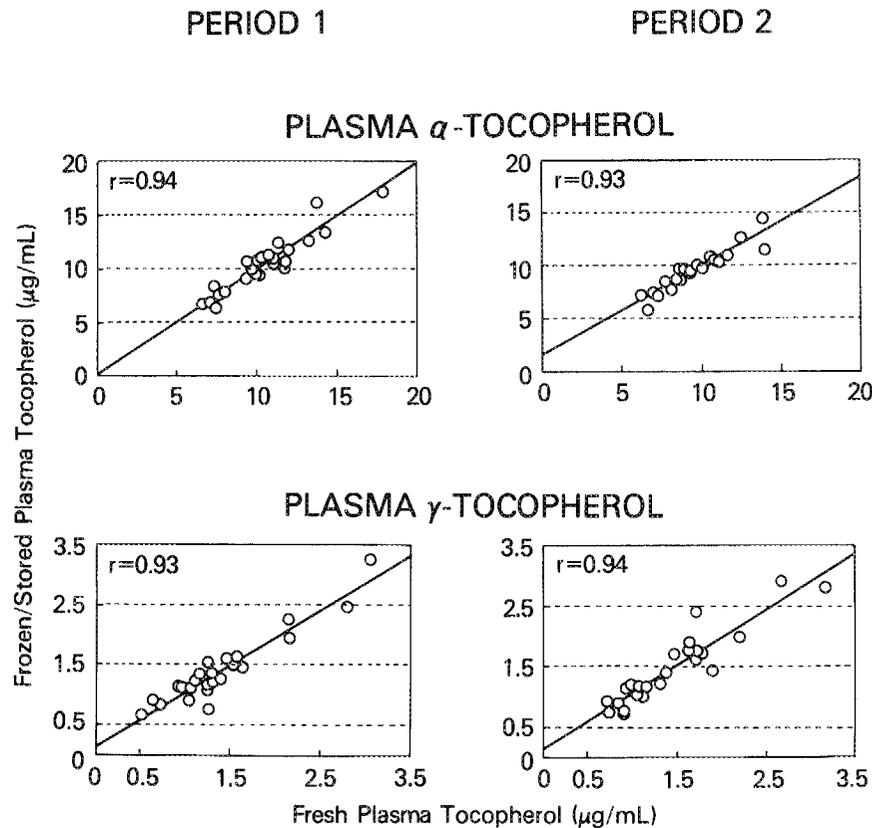


FIG. 1. Correlations of  $\alpha$ - and  $\gamma$ -tocopherol levels in frozen/stored vs fresh plasma for two blood collection periods.

2:  $r = 0.81$ ,  $P < 0.001$ ), due largely to the narrow range of HDL  $\alpha$ -tocopherol values.

**Distribution of  $\alpha$ -tocopherol between LDL-VLDL and HDL.** Distribution of  $\alpha$ -tocopherol between the LDL-VLDL and the HDL fractions is shown in Table 2. With the exception of the LDL-VLDL fraction for period 1, the percentage of tocopherol carried by lipoprotein fractions did not differ for fresh and frozen/stored samples. The higher percentage distribution of  $\alpha$ -tocopherol observed in period 1 for LDL-VLDL from frozen/stored plasma (62 vs 57%) was probably a function of the improved recovery (99 vs 95%) associated with the frozen/stored samples, as discussed below, rather than a direct effect of freezing and storage.

**ApoB in column fractions.** ApoB was confined exclusively to the LDL-VLDL fraction from each of the eight frozen/stored plasma samples analyzed.

## DISCUSSION

As anticipated, plasma  $\alpha$ - and  $\gamma$ -tocopherol were stable under the storage conditions of this study. Craft *et al.* (10) reported that  $\alpha$ -tocopherol in heparinized plasma was stable for at least 15 months at  $-20^{\circ}\text{C}$  and that purging plasma samples with nitrogen gas prior to storage had no detectable beneficial effects. Driskell *et al.* (11), using

a single-donor pool of human serum, found that  $\alpha$ -tocopherol was stable for 16 months when stored at  $-20^{\circ}\text{C}$ . Furthermore,  $\alpha$ -tocopherol levels were unaffected by 17 cycles of freezing and thawing. In the present study, all plasma samples contained EDTA as an added precaution against oxidative degradation of lipoprotein components (12) including loss of  $\alpha$ -tocopherol (7).

The suitability of frozen plasma for determination of lipoprotein tocopherol levels appears to depend more on the stability of the lipoproteins than the stability of tocopherol. Several studies have been conducted to investigate the influence of freezing and storage of plasma on stability of lipoproteins as judged by recovery of lipoprotein cholesterol following ultracentrifugation. Two reports (13,14) concluded that lipoproteins are rapidly degraded by freezing, but others (16,17) concluded that decomposition of lipoproteins in frozen plasma was negligible for at least several months.

Frozen plasma has certain advantages over fresh plasma for lipoprotein fractionation and  $\alpha$ -tocopherol analysis. Use of frozen replicate samples provides the opportunity to repeat chromatography and analysis of samples when recovery of  $\alpha$ -tocopherol in lipoprotein fractions is incomplete. Storage also provides the convenience of pacing sample analysis. This can be important when large numbers of samples are to be analyzed; or, as frequently

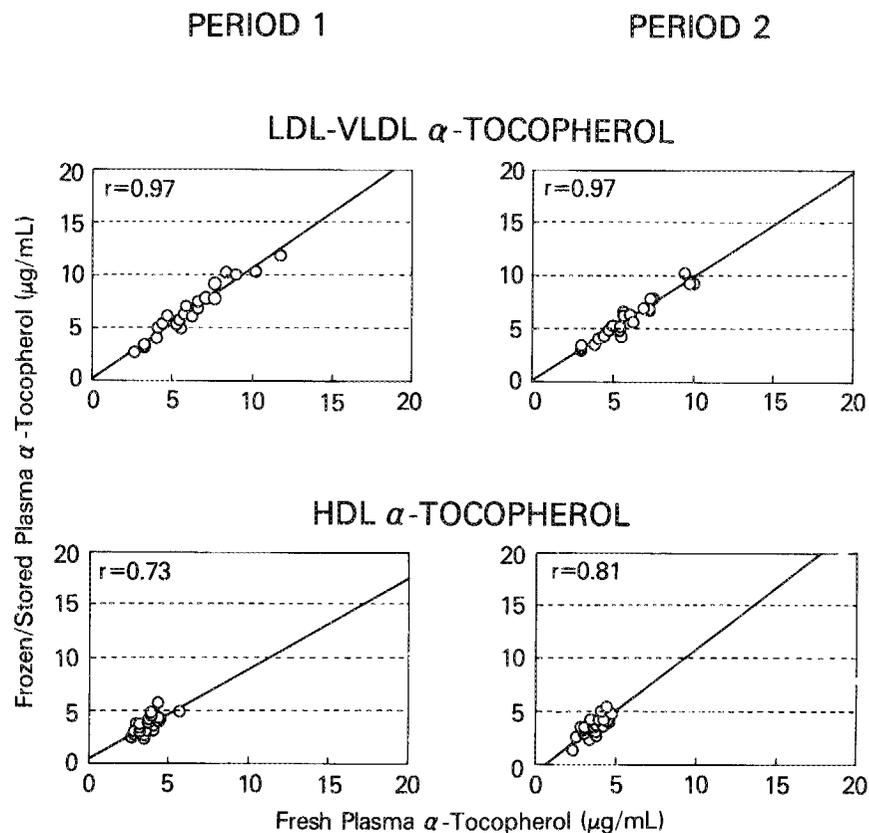


FIG. 2. Correlations of  $\alpha$ -tocopherol levels in lipoprotein fractions separated by affinity chromatography from frozen/stored vs fresh plasma for two blood collection periods.

TABLE 2

Percent of Whole Plasma  $\alpha$ -Tocopherol Recovered in LDL-VLDL and HDL Fractions Separated by Affinity Chromatography Using Fresh or Frozen/Stored Plasma<sup>a</sup>

	Fresh	Frozen/ stored <sup>b</sup>	Paired t-test	
			DIF $\pm$ SE <sup>c</sup>	p <sup>d</sup>
Percent				
Period 1				
LDL-VLDL	57 $\pm$ 1.8	62 $\pm$ 2.0	-5 $\pm$ 1.3	<0.001
HDL	38 $\pm$ 1.7	37 $\pm$ 2.1	1 $\pm$ 1.0	NS
Recovery	95 $\pm$ 1.2	99 $\pm$ 1.2	-4 $\pm$ 1.7	0.019
Period 2				
LDL-VLDL	63 $\pm$ 1.9	62 $\pm$ 2.1	<1 $\pm$ 1.3	NS
HDL	40 $\pm$ 1.6	38 $\pm$ 2.1	1 $\pm$ 1.1	NS
Recovery	103 $\pm$ 1.2	100 $\pm$ 0.9	2 $\pm$ 1.8	NS

<sup>a</sup>Mean  $\pm$  SEM (N = 24).

<sup>b</sup>Samples stored at  $-20^{\circ}\text{C}$  for six weeks prior to lipoprotein fractionation and tocopherol analysis.

<sup>c</sup>Mean difference and standard error (SE) of the difference.

<sup>d</sup>Probability determined by paired t-test,  $P > 0.10$  = nonsignificant (NS).

required, when lipids, proteins, or other components of column fractions are to be concurrently measured. Use of frozen plasma also provides the opportunity to analyze, in one run, plasma samples collected at different times from a single donor.

In spite of such advantages, most investigators have chosen to use fresh plasma, and thus avoid the possibility that physical forces involved in freezing and thawing could alter lipoprotein integrity. In the present study, plasma that had been frozen at  $-20^{\circ}\text{C}$  for six weeks was examined for evidence of lipoprotein decomposition. Ineffective binding of apoB to heparin affinity columns and loss of LDL tocoferol were not evident as might be expected if LDL particles had been substantially altered.

Freezing and storage of plasma did not appear to disrupt the binding of apoB-containing lipoproteins to affinity columns. If the affinity of apoB for the heparin ligand had been lost, then apoB should have been found in the unbound (HDL) fraction. Yet, no apoB was detected in that fraction for the eight plasma samples analyzed.

Furthermore,  $\alpha$ -tocopherol content of lipoproteins was not decreased due to fractionating plasma that had been subjected to a single freeze-thaw cycle. Although a difference in the  $\alpha$ -tocopherol content of LDL-VLDL was observed for period 1, the lower  $\alpha$ -tocopherol value was from the LDL-VLDL fraction that was produced from fresh plasma. It is likely that this lower value was the result of an unexplained 5% lower recovery of LDL-VLDL from fresh plasma during period 1. That conclusion is supported by the  $\alpha$ -tocopherol values for LDL-VLDL during period 2. There, the difference between mean  $\alpha$ -tocopherol values for fresh and frozen/stored LDL-VLDL was negligible ( $-0.01 \pm 0.10$ ,  $P = 0.900$ ). This illustrates the importance of complete recovery for accurate determination of lipoprotein tocoferol levels. Our 95% recovery from period 1, fresh plasma, was associated with a 9% decrease in the mean value for LDL-VLDL  $\alpha$ -tocopherol as compared to frozen plasma where recovery averaged 99%. Yet, recoveries as high as 95% are generally not achieved using traditional methods of lipoprotein separation such as ultracentrifugation and gel filtration (1,2).

Because our observations were limited to a single storage temperature and to a relatively brief period of storage, they cannot be generalized to all conditions of low-temperature storage. Additionally, it is not known whether the relatively low lipid levels of the plasma samples used in this study influenced the outcome. In hypertriglyceridemic subjects VLDL can carry an unusually large proportion of  $\alpha$ -tocopherol (3), and decay of VLDL and chylomicrons appears to be more pronounced in plasma samples with relatively high concentrations of these lipoproteins (15). Since fasting, normolipemic subjects carry only a minor proportion of  $\alpha$ -tocopherol in VLDL (1-3),  $\alpha$ -tocopherol contents of the LDL-VLDL fractions from this study predominately reflect  $\alpha$ -tocopherol contents of LDL.

Measuring  $\alpha$ -tocopherol content of LDL may prove useful for assessing the degree of protection this lipophilic antioxidant provides against certain oxidative processes. Recent studies have implicated oxidatively modified LDL as a promoter of atherosclerosis (22,23). *In vitro*, modified LDL are chemotactic for human monocytes (22) and promote lipid accumulation in macrophages (5,24). They are also toxic to cells in culture (25-27). Yet, it has been

clearly demonstrated that oxidative modification of LDL, *in vitro*, is effectively inhibited by  $\alpha$ -tocopherol (7,26,27). If these processes are operative *in vivo*, then the  $\alpha$ -tocopherol carried by LDL could play a significant role in protecting this lipoprotein from oxidative modification.

We have previously described a rapid method for determining  $\alpha$ -tocopherol levels of plasma lipoproteins. The present study expands the versatility of that method by demonstrating that plasma can be stored at  $-20^{\circ}\text{C}$  for at least six weeks prior to lipoprotein fractionation by affinity chromatography without loss of  $\alpha$ -tocopherol from either LDL-VLDL or HDL fractions. This simplified and convenient method for assessing  $\alpha$ -tocopherol content of LDL may have practical application for assessing susceptibility to oxidative stresses.

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