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Validation of Analytical Methods for Plasma Total Antioxidant Capacity by Comparing with Urinary 8-Isoprostane Level

Sang Gil Lee[1], Taoran Wang[1], Terrence M. Vance[1], Patrice Hurbert[1], Dae-Ok Kim[2], Sung I. Koo[1#], and Ock K. Chun[1*]

[1]*Department of Nutritional Sciences, University of Connecticut, Storrs, CT 06269-4017, USA*
[2]*Department of Food Science and Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea*

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*Corresponding author
Phone: +1-860-486-6275;
Fax: +1-860-486-4876;
E-mail: ock.chun@uconn.edu

[#]Invited Scholar at Department of Food Science and Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea

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Although several analytical methods for measuring total antioxidant capacity (TAC) have been applied to biological samples, there were often dissimilar results due to the different principles of methods applied. Thus, this study aimed to validate four conventional analytical methods for measuring plasma TAC, including the ABTS assay, DPPH assay, FRAP assay, and ORAC assay, by comparing with urinary 8-isoprostane concentration. In addition, TAC results were compared with antioxidant enzyme activities including superoxide dismutase (SOD) and glutathione peroxidase in erythrocyte, and catalase in plasma. Plasma TAC measure by ABTS assay was strongly correlated with the result by FRAP assay. Plasma TAC by FRAP and ORAC assays were negatively correlated with erythrocyte SOD activity. The agreement among the four TAC assay methods and 8-isoprostane was determined using 95% prediction limits of linear regression, expressed as the mean of 8-isoprostane ± 95% prediction limits. The ABTS method better agreed with 8-isoprostane than the other methods, demonstrating narrow prediction of limits. Furthermore, only plasma TAC determined by the ABTS assay was inversely correlated with urinary 8-isoprostane (r = −0.35, p < 0.05). In summary, the ABTS assay would be an appropriate method to measure overall plasma antioxidant capacity and predict the body's antioxidant status.

Keywords: Total antioxidant capacity, ABTS assay, FRAP assay, 8-isoprostane, superoxide dismutase

Introduction

Reactive oxygen species (ROS) has been known to be involved in a variety of cellular processes, including cell signaling and host defense system [1–3]. In spite of those important physiological roles, excessive ROS, which is considered oxidative stress, act as deleterious molecules [4]. Oxidative stress causes the damage of cells by reacting with various cellular components, including carbohydrate, proteins, lipids, and DNA [4]. This cellular damage by oxidative stress is associated with the development of various chronic diseases, such as cancer, cardiovascular disease (CVD), type 2 diabetes, osteoarthritis, and Alzheimer's disease [5–7].

A balance between ROS and antioxidants is essential to mitigate the damage from oxidative stress and to reduce the risk of related chronic diseases. Different types of antioxidants, in vivo and in vitro, are involved in the regulatory system to maintain appropriate levels of ROS, including (i) endogenous antioxidants such as albumin, bilirubin, glutathione (GSH), and uric acid; (ii) antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), and NAD(P)H: quinone oxidoreductase (NQO1); and (iii) dietary antioxidants, including vitamins C and E, carotenoids, and various polyphenol compounds.

Since the in vivo antioxidant level is very critical for a health condition, measuring the plasma antioxidant capacity is considered a possible surrogate to predict the health condition [8]. Various analytical methods have been developed

to measure total antioxidant capacity (TAC), including the 2,2′-azino-*bis*-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, and oxygen radical absorbance capacity (ORAC) assay. These methods have been conventionally used owing to their easy and fast characteristics [9]. The principle of the conventional TAC assay methods is based on the radical scavenging activity and redox potential of antioxidants [9, 10]. Those methods for TAC measurement were originally developed to measure the TAC of foods and nutrients. Unlike foods and nutrients, biological fluids contain not only dietary antioxidants but also various types of endogenous antioxidant enzymes, dietary antioxidants, and even oxidative products [11, 12]. For this reason, various approaches measuring the TAC of biological samples have been tried. In practice, not only using conventional TAC assay methods, but measuring antioxidant enzyme levels in blood has been frequently used to determine the TAC level in vivo [13]. In addition, measuring various oxidative products is also a popular tool for TAC measurement [13]. Since various analytical methods are eligible for TAC measurement of biological samples, the compatibility and validity of the methods need to be evaluated.

Although the compatibility of different analytical methods has been evaluated in previous studies for foods and nutrients, their compatibility with biological fluids is still little examined [14]. In addition, comparison of different TAC assay methods for the biological sample would have some limitations due to the lack of a golden standard. 8-Isoprostane has been detected in plasma and urine of humans, and elevated 8-isoprostane levels have been found in various health conditions in which oxidative stress increased [15]. Therefore, it has been proposed as a reliable marker of antioxidant deficiency and oxidative stress.

The aim of this study was to evaluate the compatibility of the four conventional TAC assay methods (*i.e.*, ABTS, DPPH, FRAP, and ORAC), using human plasma. This study also tested the validity of the assays in predicting the body's oxidative stress status by comparing the results with urinary 8-isoprostane levels.

Materials and Methods

Chemicals

ABTS, 2 2′-azo*bis*(2-amidinopropane) dihydrochloride (AAPH), methanol, DPPH, 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), ferric chloride, sodium acetate, glacial acetic acid, sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, sodium phosphate monobasic, fluorescein, Trolox, and L-ascorbic acid were purchased from Sigma-Aldrich (USA).

Sample Preparation

Blood samples after overnight fasting and urine samples collected during the overnight period, from 45 subjects who participated in an intervention study on the effects of a chokeberry supplement in former smokers, were used to measure TAC by the ABTS, DPPH, FRAP, and ORAC assays. Plasma samples were isolated from baseline blood samples in the study by centrifugation for 15 min at 2,000 ×*g*. Urine samples corresponding to plasma samples were used to investigate the agreement between TAC by each of the four methods with urinary 8-isoprostane concentrations. After the plasma was collected, erythrocytes were mixed with 4 volumes of cold water and centrifuged at 10,000 ×*g* for 15 min to prepare erythrocyte lysate for antioxidant enzyme measurement. All procedures were approved by the Institutional Review Board committee of the University of Connecticut.

ABTS Radical Scavenging Assay

Fresh ABTS radical solution was prepared by dissolving 1.0 mM AAPH and 2.5 mM ABTS in 100 ml of phosphate buffer solution (PBS), pH 7.4, and allowing the mixture to react for 30 min at 70°C. To measure TAC of plasma sample, three times diluted plasma samples (5 µl) were reacted with the ABTS radical working solution (245 µl) in a 96-well plate at 37°C for 10 min and the absorbance was measured using a Synergy 2 microplate reader (BioTek, USA) at 734 nm. TAC was expressed as mg vitamin C equivalent /l (mg VCE/l).

DPPH Radical Scavenging Assay

Fresh DPPH radical solution was prepared by dissolving 1.0 mM of DPPH in 200 ml of 80% (v/v) methanol. Plasma samples were diluted with 80% (v/v) methanol and centrifuged for 5 min at 14,000 ×*g* to remove precipitated protein. The supernatant (5 µl) was reacted with DPPH working solution (245 µl) in a 96-well plate for 30 min and the absorbance was measured using a microplate reader at 517 nm. TAC was expressed as mg VCE/l.

FRAP Assay

Plasma samples diluted four times with PBS (5 µl) were reacted with 245 µl of the FRAP working solution in a 96-well plate prepared with a slight modification of the method developed by Miller *et al*. [16]. The absorbance was measured at 593 nm using a microplate reader. All reagents for the FRAP assay were prepared using chelated water. TAC was expressed as mg trolox equivalent/l (mg TE/l).

ORAC Assay

Plasma samples diluted 300 times using DDW (25 µl) were allowed to react with the ORAC working solution (150 µl) as previously described [17]. After adding AAPH for peroxyl radical generation, the fluorescence was measured for 80 min to determine

the area under curve (AUC) at 485 nm as excitation and at 520 nm as emission. TAC was expressed as mg TE/l.

Measuring Antioxidant Enzyme Level

Colorimetric assay kits purchased from Cayman (USA) were used to measure the activity of SOD (Catalog No. 706002) and GPX (Catalog No. 703102) in erythrocytes, and plasma CAT activity (Catalog No. 707002), according to the manufacturer's instructions. The amount of SOD enzyme to exhibit 50% dismutation of the superoxide radical, which was generated from a tetrazolium salt by xanthine oxidase and hypoxanthine, was defined as SOD activity. The GPX activity was measured indirectly by a coupled reaction with glutathione reductase on the basis of recycling oxidized glutathione to reduced glutathione by glutathione reductase (GR) and NADPH. For CAT activity, the assay kits utilized the peroxidatic function of CAT ($H_2O_2 + AH_2 \rightarrow A + 2H_2O$), since the reaction of the CAT with methanol in the presence of H_2O_2 produces formaldehyde, which is calorimetrically measured with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen.

Measuring Urinary 8-Isoprostane Concentrations

Urinary 8-isoprostane level was measured using ELISA (Cayman, USA) following the manufacturer's instructions (Catalog No. 516351). The assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase (AChE) conjugate reagent in the ELISA kit (8-isoprostane Tracer). The concentration of the 8-isoprostane Tracer is held constant while the concentration of 8-isoprostane varies. Thus, the concentration of 8-isoprostane in the well was measured, since it is inversely proportional to the amount of 8-isoprostane Tracer that can bind to the rabbit antiserum during the incubation. The 8-isoprostane level was adjusted using the urinary creatinine level and expressed as ng/mmol creatinine.

Statistical Analysis

SAS was used to evaluate the compatibility between plasma TAC measured by each assay and 8-isoprostane. In order to validate the analytical methods for plasma TAC, the agreement of the four TAC assay methods with urinary 8-isoprostane level was determined by comparing the mean and 95% prediction limits for 8-isoprostane from linear regression; the narrower the prediction limits, the better agreement with the urinary 8-isoprostane level [18]. The 95% prediction limits were calculated as $\mathbf{1.96 \times \sqrt{\{MSE \times (1 + 1/45)\}}}$, where MSE is the mean squared error from linear regression [18, 19]. Correlations between the TAC assay methods, antioxidant enzyme activities, and urinary 8-isoprostane level were analyzed using the Pearson Correlation method.

Results and Discussion

Correlation among Different TAC Assay Methods

Table 1 shows a positive correlation between the ABTS assay and the FRAP assay as shown in many other studies, including our previous study [17, 20, 21]. This strong correlation between the ABTS and FRAP assays would be in part attributed to plasma uric acid and ascorbic acid, which contribute to the TAC measured by those two methods [12, 20, 22]. Although the excessive level of plasma uric acid causes the increase in CVD risk, the normal range of plasma uric acid has a beneficial role due to its antioxidant activity [23]. Ascorbic acid is a well-known important radical scavenger in plasma [24]. It has been reported that uric acid and ascorbic acid can contribute to plasma TAC levels by ABTS assay of up to 65% and 24%, respectively [24]. Therefore, uric acid and ascorbic acid would have mainly affected the result of TAC measured by ABTS assay. Regarding the FRAP assay, although it has been known as a method to measure the reducing power of antioxidants, the method practically measures the antioxidants that have reducing power, including uric acid and ascorbic acid. Indeed, the reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ by reducing agents is defined as antioxidant capacity in the FRAP assay. Ironically, however, Fe^{2+} is considered a strong pro-oxidant to generate superoxide radical ($OH\cdot$) from H_2O_2 in the Fenton reaction [9]. Therefore, results of the FRAP assay assume that the antioxidants that can reduce Fe^{3+}-TPTZ also have antioxidant capacity against Fe^{2+}. Uric acid and ascorbic acid are typical compounds exerting both abilities, and thus the FRAP assay may also be profoundly dependent on plasma uric acid and ascorbic acid levels [25]. This would be the reason for the strong correlation between the ABTS and FRAP assays.

The ABTS assay and ORAC assay results were not significantly correlated in our study. The reason for the lack of correlation would be due to the insufficient reaction time in the ABTS assay. One study determined the correlation between plasma ORAC values with a 70 min reaction time and trolox equivalent antioxidant capacity (TEAC) results at different time points measured by ABTS assay. In this study, a sufficient reaction time in the ABTS assay (30 min) led to a significant correlation with the ORAC assay. However, TEAC values with less than 5 min reaction did not show significant correlation with ORAC values [26]. Although the present study measured the antioxidant capacity of plasma against the ABTS radical after 10 min reaction, the results might not have been sufficient to be commensurate with the ORAC assay, which tracks the whole reaction (80 min) between a fluorescence radical and plasma using the AUC. Thus, it is speculated that the end point for the ABTS assay and the reaction time for the AUC in the ORAC assay would be critical factors for a significant

Table 1. Correlations between plasma total antioxidant capacity (TAC) assay methods, erythrocyte antioxidant enzyme activities, and urinary 8-isoprostane level[a,d].

	TAC by ABTS assay	TAC by DPPH assay	TAC by FRAP assay	TAC by ORAC assay	CAT activity	GPX activity	SOD activity	Urinary 8-isoprostane
TAC by ABTS assay[b] (mg VCE/l)	1.00	0.28 (0.07)	**0.64*** ($p < 0.0001$)**	-0.03 (0.82)	0.07 (0.65)	0.21 (0.17)	-0.10 (0.50)	**-0.35* ($p < 0.05$)**
TAC by DPPH assay (mg VCE/l)		1.00	0.22 (0.15)	-0.117 (0.44)	0.27 (0.08)	-0.06 (0.71)	0.23 (0.12)	-0.26 (0.08)
TAC by FRAP assay[b] (mg TE/l)			1.00	0.21 (0.18)	0.20 (0.18)	0.14 (0.37)	**-0.38* ($p < 0.05$)**	-0.07 (0.66)
TAC by ORAC assay (mg TE/l)				1.00	0.12 (0.45)	0.006 (0.97)	**-0.43* ($p < 0.01$)**	0.21 (0.17)
CAT activity (nmol/min/ml)					1.00	-0.12 (0.46)	-0.22 (0.13)	-0.07 (0.64)
GPX activity (nmol/min/ml)						1.00	-0.007 (0.98)	0.21 (0.16)
SOD activity (unit/ml)							1.00	**-0.33* ($p < 0.05$)**
Urinary 8-isoprostane[c] (ng/mmol creatinine)								1.00

[a]CAT, GPX, and SOD stand for catalase, glutathione peroxidase, and superoxide dismutase, respectively.

[b]VCE and TE stand for vitamin C equivalent and trolox equivalent, respectively.

[c]Urinary 8-isoprostane level was adjusted with urinary creatinine concentration.

[d]The correlations in bold indicate statistical significance ($p < 0.05$).

correlation between the two methods.

In our study, the DPPH assay did not appear to be appropriate to measure plasma TAC because of protein precipitation by the DPPH radical solution. The solvating power of water for a charged, hydrophilic protein molecule is decreased as the concentration of organic solvent increases. The DPPH assay uses 80% aqueous methanol to make a DPPH radical solution. This methanol solution caused protein precipitation, which confounded the OD value of the radical-plasma mixture. Although the sample was centrifuged at 14,000 ×*g* before measuring OD after 30 min reaction, the precipitation occurred even after centrifugation. In practice, DPPH was widely used to measure the TAC of hydrophobic samples. It has been known that plasma proteins can contribute to the plasma TAC at around 10–50% [24]. Therefore, removal of plasma protein can also alter the TAC of plasma, which can disturb the accurate TAC measurement.

In contrast to our result, one study showed a significant correlation between the ORAC and FRAP assays, but there was no correlation between the ORAC and ABTS assays, and the FRAP and ABTS assays [27]. This discrepancy might be due to the different analytical conditions, such as experimental condition, buffer, or reagent solutions [25].

Correlation between Plasma TAC and Antioxidant Enzyme Activities

Antioxidant enzymes are one of the most important contributors to the antioxidant defense system in vivo. In particular, SOD is one of the essential antioxidant enzymes for most living organisms. SOD is the enzyme involved in the first step of a radical scavenging process. The function of SOD is catalyzing the dismutation of superoxide radicals ($O_2^{\bullet -}$). There are three types of SOD, including SOD1, SOD2, and SOD3, depending on their location in the human body. SOD3, which is called extracellular SOD, plays a major role in blood.

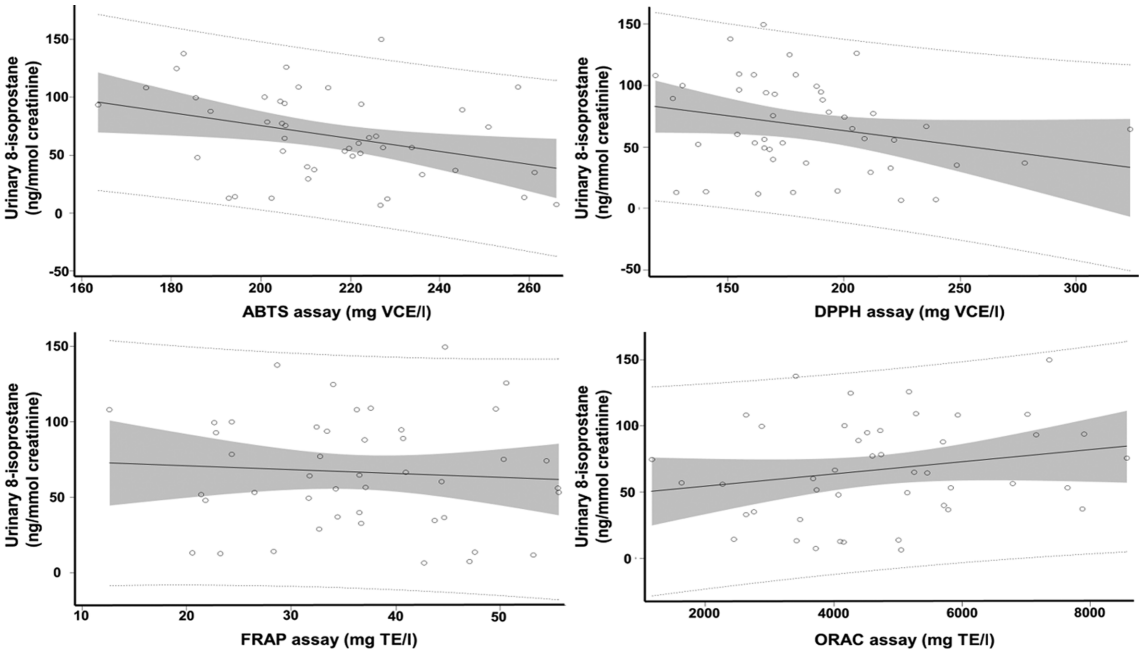
Table 1 exhibited that the TAC levels measured by the FRAP and ORAC assays were negatively correlated with erythrocyte SOD activity, but not with CAT and GPX. This inverted relationship between plasma TAC and SOD activity indicates that the enzymatic antioxidant defense system is properly operated in vivo. Another study also showed increased antioxidant enzyme activity in the condition of exerted oxidative stress [28, 29]. One study revealed higher SOD activity in patients with systemic sclerosis compared

with healthy subjects [28]. The FRAP and ORAC assays measure TAC, which reflects the status of antioxidant capacity in plasma, whereas SOD is measured for their activity. A lower antioxidant status can activate the antioxidant defense system, which augments SOD activity. Therefore, our result demonstrates that the antioxidant defense system can be stimulated, corresponding to the antioxidant status in vivo.

SOD activity was negatively correlated with the 8-isoprostane level, although SOD was also negatively correlated with the plasma TAC measured by the FRAP and ORAC methods. Other studies also showed that antioxidant enzyme activity, including SOD, was inversely correlated with the urinary 8-isoprostane level and other lipid peroxidation products [8, 30]. This inverted correlation would be attributed to the prevention of 8-isoprostane generation by the antioxidant defense system in vivo. Indeed, other studies showed that SOD plays a critical role in inhibiting lipid peroxidation, which is also an important procedure for the generation of 8-isoprostane [22]. Therefore, our results demonstrate that the antioxidant defense system can control the generation of oxidative stress corresponding to the antioxidant status in vivo.

Validation of TAC Assay Methods by Comparing with Urinary 8-Isoprostane Level

Various biomarkers are used to measure the in vivo antioxidant status or oxidative stress level, including plasma and erythrocyte antioxidant enzymes and oxidation products. Among those, the urinary 8-isoprostane level has been considered a golden standard for the measurement of oxidative stress [15]. One study showed that plasma TAC measured by the ABTS assay was significantly decreased after exercise, whereas the urinary 8-isoprostane level adjusted with the creatinine level was significantly increased [31]. Among the four TAC assay methods, only the ABTS assay was negatively correlated with the urinary 8-isoprostane level. This result may indicate that the ABTS



	ABTS assay	DPPH assay	FRAP assay	ORAC assay
Mean 8-isoprostane ± 95% Prediction limits (ng/mmol creatinine)	66.8 ± 70.2	66.8 ± 72.4	66.8 ± 74.8	66.8 ± 73.3

Fig. 1. Result of the prediction interval of the mean of the total antioxidant capacity (TAC) measured by the ABTS, DPPH, FRAP, and ORAC assays corresponding to the 8-isoprostane level (mean 8-isoprostane ± 95% prediction limits).
(**A**) ABTS assay to 8-isoprostane level, (**B**) DPPH to 8-isoprostane level, (**C**) FRAP assay to 8-isoprostane level, and (**D**) ORAC assay to 8-isoprostane level.

assay has a better sensitivity to the systemic antioxidant status compared with the other methods. One study reported that a high intake of fruit and vegetable reduced the urinary 8-isoprostane level, which revealed that plasma dietary antioxidants can prevent the production of 8-isoprostane [32]. Since ABTS is conventionally used to measure the antioxidant capacity of dietary antioxidants in fruits and vegetables, ABTS would be appropriate to cover the TAC of exogenous antioxidants in plasma, too. Indeed, our previous study demonstrated that the ABTS assay was an appropriate method to measure the correlation between dietary TAC and plasma TAC [21]. As shown in Fig. 1, the result of linear regression of the ABTS assay had the narrowest 95% prediction interval among the four TAC assay methods. The prediction interval of the mean of measured TAC via the ABTS, DPPH, FRAP, and ORAC assays corresponding to the 8-isoprostane level were 66.8 ± 70.2 ng/mmol creatinine for ABTS, 66.8 ± 72.4 ng/mmol creatinine for DPPH, 66.8 ± 74.8 ng/mmol creatinine for FRAP, and 66.8 ± 73.3 ng/mmol creatinine for ORAC assays, respectively. This result indicates that the ABTS method is the most compatible with the urinary 8-isoprostane assay [18].

On the other hand, the FRAP assay did not show a significant correlation with urinary 8-isoprostane level, although the ABTS and FRAP assays were highly correlated. It might be due to the limitation of the FRAP assay for measuring the antioxidant capacity of thiol antioxidants, including GSH and albumin [9]. The reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ as the antioxidant capacity in the FRAP assay does not efficiently occur by those antioxidants [9]. Thus, unlike the ABTS assay, this limitation may have caused nonsignificant correlation between the FRAP assay and the urinary 8-isoprostane level.

To our best knowledge, the present study is the first to use the urinary 8-isoprostane level to validate conventional TAC analytical methods in plasma. The ABTS and FRAP assays were strongly correlated to each other. However, only the ABTS assay showed the most compatibility with the urinary 8-isoprostane level. This result indicates that the ABTS assay can be applied to measure TAC in vivo. Although the FRAP assay and ORAC assay were not correlated with the 8-isoprostane results, they were significantly correlated with SOD activity. As the SOD activity was also negatively correlated with the urinary 8-isoprostane level, it is evident that antioxidant enzymes can be activated in response to the antioxidant status, which regulates the production of oxidative stress.

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