Published in final edited form as:

Int J Sport Nutr Exerc Metab. 2010 February ; 20(1): 2–14.

Experimental Guidelines for Studies Designed to Investigate the Impact of Antioxidant Supplementation on Exercise Performance

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Abstract

Research interest in the effects of antioxidants on exercise-induced oxidative stress and human performance continues to grow as new scientists enter this field. Consequently, there is a need to establish an acceptable set of criteria for monitoring antioxidant capacity and oxidative damage in tissues. Numerous reports have described a wide range of assays to detect both antioxidant capacity and oxidative damage to biomolecules, but many techniques are not appropriate in all experimental conditions. Here, the authors present guidelines for selecting and interpreting methods that can be used by scientists to investigate the impact of antioxidants on both exercise performance and the redox status of tissues. Moreover, these guidelines will be useful for reviewers who are assigned the task of evaluating studies on this topic. The set of guidelines contained in this report is not designed to be a strict set of rules, because often the appropriate procedures depend on the question being addressed and the experimental model. Furthermore, because no individual assay is guaranteed to be the most appropriate in every experimental situation, the authors strongly recommend using multiple assays to verify a change in biomarkers of oxidative stress or redox balance.

Keywords

radicals; free radicals; reactive oxygen species; oxidative stress; redox

The observation that contracting skeletal muscles produce free radicals (hereafter referred to as radicals) was first reported in 1982 (Davies, Quintanilha, Brooks, & Packer, 1982). Many studies have since confirmed that contracting muscles produce radicals and other reactive oxygen species (ROS). Moreover, it is now established that the levels of ROS in skeletal muscle play a critical role in regulating force production. Indeed, an optimal redox balance exists in muscle whereby the contractile apparatus generates the highest force production. Furthermore, it is clear that high-intensity prolonged exercise promotes muscle ROS production, resulting in oxidative damage and impaired muscle function (Powers & Jackson, 2008).

Evidence that contracting skeletal muscles produce radicals and that radicals can promote fatigue and injury in skeletal muscle has stimulated researchers to study the ability of antioxidants to prevent both exercise-induced oxidative damage and muscle fatigue (Powers & Sen, 2000; Powers, DeRuisseau, Quindry, & Hamilton, 2004). On the surface, these types of studies appear to be straightforward. However, the successful design and completion of rigorous studies in this field is complicated by many factors. For example, what are the essential criteria for establishing the presence of oxidative damage in cells or tissue? This appears to be

a direct question, but, unfortunately, the answer to this query often presents a “moving target” for researchers who think they have met the criteria only to discover that a reviewer has a different view. Likewise, as a reviewer, it becomes a burden to repeatedly raise the same questions to authors about the failure to meet the criteria to document the presence of oxidative damage in tissues. Accordingly, it is important to establish a set of guiding principles that can be used by investigators to design and interpret their experiments. Likewise, these guidelines should also prove helpful to reviewers assigned the task of evaluating studies on this topic.

Therefore, the objective of this commentary is to provide a set of contemporary and rigorous guidelines for investigating the effects of antioxidant supplementation on exercise performance and the redox status of tissues. This report will critique many of the assays available to quantify biomarkers of oxidative damage and assays of antioxidant capacity. Furthermore, it will provide suggested guidelines on other key aspects of planning and interpreting experiments in this field. We begin with a discussion of the evolving definition of oxidative stress.

Oxidative Stress Redefined

The term *oxidative stress* was first defined in the 1980s as “a disturbance in the pro-oxidant-antioxidant balance in favor of the former” (Sies, 1985). Although this meaning has been widely accepted for over 2 decades, the definition of oxidative stress has been challenged. Indeed, because of the complexity associated with the assessment of cellular redox balance, it has been argued that the term *oxidative stress* defies a simple pro-oxidant versus antioxidant definition (Azzi, Davies, & Kelly, 2004; Jones, 2006). Several new definitions of oxidative stress have appeared in the literature, including the suggestion that it should be redefined as “a disruption of redox signaling and control” (Jones, 2006, p. 1865). Using this definition, the assessment of tissue redox status would become the defining measure of whether oxidative stress is present. Regardless of whether this new definition gains acceptance, it can be anticipated that the description of oxidative stress will undergo future modifications as the field of redox biology advances. Therefore, investigators using the term *oxidative stress* should clearly define the term in the framework of their research. In the context of this report, we will define oxidative stress as a disturbance in the redox balance in cells in favor of oxidants, with this imbalance resulting in oxidative damage to cellular components.

Guidelines for Experiments Investigating Antioxidants and Exercise Performance

Several important points should be stressed as we establish guidelines for the appropriate methods to study the effect of antioxidants on exercise performance. First, in every experimental situation, there are no absolute criteria for evaluating total antioxidant capacity in all tissues or determining whether an antioxidant is capable of protecting against exercise-induced oxidative stress. This is because some assays are inappropriate or may not work in all experimental conditions. In addition, there is no single biomarker of oxidative stress or damage that can be measured to accurately assess whether the antioxidant of interest protected against oxidative stress or damage. Therefore, several reliable biomarkers of oxidative stress or damage should be used. Finally, our proposed guidelines will undoubtedly evolve as new techniques emerge that supersede current procedures. Nonetheless, it is important to establish guidelines for the current acceptable assays that can evaluate oxidative stress and antioxidant capacity. We begin with brief comments on the choice of an exercise test to evaluate human performance.

Laboratory Tests to Evaluate Human Performance

Selecting the appropriate exercise test becomes a critical consideration in an experimental design if the objective of the study is to determine the effects of antioxidant supplementation

on exercise performance. Over the years, many studies have evaluated human performance in the laboratory, and there has been extensive debate about the correct use of exercise protocols. Clearly, the use of accurate performance exercise tests is needed to achieve the ultimate goal of delineating links between oxidative stress and human performance. A detailed discussion of all the considerations involved in selecting a performance test is beyond the scope of this report, but a few comments on the importance of choosing the appropriate exercise test for use in antioxidant studies is warranted. In selecting an exercise test to assess changes in performance between experimental treatments, three aspects of the test must be considered: validity, reliability, and sensitivity (Currell & Jeukendrup, 2008).

A valid exercise test is one that closely resembles the performance of the event being simulated. When investigating endurance-race events (e.g., running or cycling), the most common exercise protocols are a time-to-exhaustion test (i.e., test endpoint would be the failure to maintain a defined power output) and a time-trial test (i.e., target endpoint would be the distance covered). It has been argued that time trials have greater validity than time-to-exhaustion tests because they simulate the actual race event and are highly correlated with actual performance in the athletic event (Currell & Jeukendrup, 2008; Laursen, Francis, Abbiss, Newton, & Nosaka, 2007).

Reliability is the lack of variation during the test protocol. Obviously, choosing an exercise test that is reliable is a requirement for any study designed to determine the effects of an antioxidant supplement on exercise performance. Previous studies reveal that time-to-exhaustion protocols have a coefficient of variation of >10%, whereas time trials are more reliable, with a coefficient of variation of <5% (Currell & Jeukendrup, 2008).

A sensitive exercise protocol is one that is capable of detecting small differences in performance. This is important because the difference between finishing first or second in endurance events is often small (Currell & Jeukendrup, 2008). Therefore, it is critical to select a sensitive exercise test that is capable of detecting small differences in exercise performance.

In conclusion, careful selection of the appropriate exercise test is a critical consideration in planning experiments to determine the impact of antioxidants on performance. Successful completion of these types of studies requires that the exercise protocol be valid, reliable, and sensitive. For a detailed review of this topic and the validity, reliability, and sensitivity of specific performance protocols, see Currell and Jeukendrup (2008); Amann, Hopkins, and Marcora (2008); and Hinckson and Hopkins (2005).

Laboratory Tests to Evaluate Endurance in Animals

Identical to performance testing in humans, the selection of the appropriate exercise test to evaluate endurance performance in animal studies is a key consideration in the plan of any experiment designed to investigate the influence of antioxidants on endurance performance. In this regard, many studies have evaluated the endurance capacity of rodents, and almost all of these have used either treadmill protocols or swimming tests. Nonetheless, there are few published reports regarding the reliability of specific exercise tests in animals. In reference to the reliability of swimming tests, one well-designed study revealed that the within-rat test-retest reliability of swim time to exhaustion was extremely variable ($r = .50$) in untrained animals (McArdle & Montoye, 1966). These findings suggest that when using untrained animals an endurance swim test is not likely to discern performance differences between groups unless the sample size is extremely large. Note, however, that the test-retest performance reliability in endurance swim tests was improved when animals were exposed to a 4-week swim-training period before the performance test ($r = .91$). This is an important consideration for investigators designing experiments that will use swimming to evaluate endurance in rodents.

In regard to the reproducibility of treadmill performance tests in rodents, there are few published reports in the literature. Nonetheless, a recent well-designed study evaluated the reliability of both endurance-exercise tests and the test–retest reliability of an incremental exercise protocol designed to determine $\text{VO}_{2\text{peak}}$ in rats running on a treadmill (Copp, Davis, Poole, & Musch, 2009). Those investigators concluded that a progressive treadmill exercise test could be used to reliably test endurance performance in untrained rats. However, this report cautioned that the test–retest variability of exercise tests could increase as more runs and elapsed time are introduced into the experimental design. Furthermore, the work by Copp et al. revealed that the test–retest reliability of $\text{VO}_{2\text{peak}}$ in rats is also reliable when using an appropriate incremental exercise test. Similar conclusions have been reached by others (Bedford, Tipton, Wilson, Oppliger, & Gisolfi, 1979).

In summary, identical to performance testing in humans, careful selection of the appropriate exercise test is a key consideration in the design of animal experiments evaluating the impact of antioxidants on exercise performance. Successful completion of any animal performance study requires that the exercise protocol be reliable. It follows that it is critical that investigators demonstrate the reliability of specific exercise tests before incorporating these tests into experiments.

Antioxidant Selection and the Importance of Dietary Control

Selecting the antioxidant compounds for study is also a key step in planning experiments to investigate whether these compounds can protect against exercise-induced oxidative stress or improve exercise performance. In selecting antioxidants for study several points should be considered. First, investigators should recognize that cellular protection against radical-mediated damage is achieved by numerous antioxidant networks working as a unit (Powers & Jackson, 2008). For example, many dietary antioxidants work synergistically to provide protection against ROS (e.g., ascorbic acid, lipoic acid, and vitamin E; Packer, Witt, & Tritschler, 1995). Therefore, a single antioxidant compound ingested in isolation may not produce the desired effects in protecting against oxidant-mediated cell damage. Indeed, it is possible that the greatest antioxidant protection may be achieved with several antioxidants working in concert, so the potential for antioxidant interaction is an important consideration in experimental design. If the investigators choose an antioxidant cocktail that produces positive benefits (e.g., improved performance), additional experiments can then be performed to uncover the contribution of individual antioxidants to the overall protection.

Moreover, when one is selecting antioxidant compounds the bioavailability of each compound is a key consideration, because the level of absorption of various antioxidant compounds differs among both individuals and species (Halliwell & Gutteridge, 2007). For example, many phenols (i.e., compounds that contain an –OH group attached to a benzene ring such as resveratrol and quercetin) found in nature are excellent antioxidants *in vitro*, but many of these molecules are poorly absorbed and exhibit low bioavailability when consumed in the diet. It follows that the limited bioavailability of these compounds would limit their usefulness as *in vivo* antioxidants (Yang, Sang, Lambert, & Lee, 2008).

In addition, the biological half-lives of different antioxidants can differ markedly. Therefore, variances in half-life between antioxidant compounds become an important consideration in planning for the temporal pattern of antioxidant administration before and during experiments. Furthermore, because supplementation with lipid-soluble antioxidants (e.g., vitamin E) requires several weeks to achieve an elevated steady-state level in biological membranes (Coombes et al., 2000; Coombes et al., 2001), selecting the appropriate duration for antioxidant supplementation becomes a crucial consideration in experimental design.

In all studies investigating the effect of antioxidant supplementation on exercise performance, careful dietary control of participants' antioxidant intake is also essential. Undeniably, it is impossible to conduct a rigorous experiment in this field without carefully evaluating the diet of the experimental participants before and during the course of the studies. For example, the dietary intake of antioxidants often varies among athletic populations, and the failure to control this variation can affect experimental findings (Farajian, Kavouras, Yannakoulia, & Sidossis, 2004; Machefer et al., 2007; Tomten & Hostmark, 2009). Moreover, when using animal models it is also important to control the diet to ensure that all animals are receiving the AIN-93g-recommended level of nutrients. In addition, it is vital to ensure that all experimental animals have received a consistent diet for an extended period (e.g., 6–8 weeks) before and during the experiments. This is important because the contents of animal diets can vary markedly between and within manufacturers. For example, diets that are high in soy protein often contain high levels of phytoestrogens that can influence the expression of numerous genes, affecting experimental outcomes (Orzechowski, Ostaszewski, Jank, & Berwid, 2002). Furthermore, to reduce variability arising from differences in bioavailability between individuals within each group it is crucial to reduce as many confounding variables as possible and to incorporate an adequate sample size to provide the appropriate statistical power.

Finally, regardless of which antioxidant compounds are chosen for study, it is essential that each compound be chemically analyzed using the appropriate analytical techniques to ensure purity. Moreover, this type of analysis is also important to ensure that the investigator can be confident of the dose that is being delivered to experimental participants. Furthermore, proper preparation and storage of antioxidant compounds should be used to prevent oxidation and degradation.

Importance of Proper Handling of Biological Material

A critical step in the successful completion of any experiment designed to discern the effect of antioxidant supplementation on exercise performance or protection against oxidative damage is properly procuring and storing the biological material before assay. A general principle that applies to any biological sample (e.g., urine, blood, skeletal muscle) is that the sample should be rapidly obtained and quickly frozen in liquid nitrogen. Freezing the material swiftly is important to limit its exposure to room air, which prevents the high PO₂ contained in room air from oxidizing lipids and proteins in the sample. After freezing, the biological material should be stored at –80 °C until assay. Finally, when preparing a muscle sample for assay, it is advisable to pulverize the frozen sample with a mortar and pestle to avoid long thawing times and prolonged exposure to room air.

The removal of skeletal-muscle samples for storage and subsequent biochemical analysis requires special mention. First, in the case of animal models, the muscle sample should be removed rapidly from the live animal at a surgical plane of anesthesia. Otherwise the cessation of breathing or the loss of blood flow to the muscle for even short periods of time results in hypoxia/ischemia that promotes increased oxidative stress in the muscle fibers before removal. Furthermore, before freezing, the muscle sample should quickly be washed in ice-cold saline to remove excess blood. This limits the number of red blood cells in the sample, which is significant because red blood cells contain antioxidants (e.g., glutathione), and contamination of muscle samples with large amounts of blood can influence several redox-related measures.

Biomarkers of Oxidative Damage

Oftentimes, radicals or ROS react with biological molecules to form a unique oxidized molecule; this damaged molecule becomes a “fingerprint” that can be used as a biomarker of oxidative damage (Dotan, Lichtenberg, & Pinchuk, 2004; Halliwell & Whiteman, 2004). These biomarkers of oxidative damage are extremely useful in studies designed to determine the

effects of dietary supplements in protecting against ROS-mediated damage to tissues. For a biomarker to be useful as a determinant of oxidative damage to tissues it should meet select criteria. In general, reliable markers of oxidative damage possess the following qualities: they are chemically unique and detectable, they are increased or decreased during periods of oxidative stress, they have relatively long half-lives, and they should not be affected by diet (Powers & Jackson, 2008). More detailed technical guidelines describing the ideal biomarker of oxidative damage have been discussed by Halliwell and Whiteman (Figure 1). Unfortunately, none of the current biomarkers of oxidative stress meets all the technical criteria outlined in Figure 1; nonetheless, some biomarkers are more reliable than others. Numerous biomarkers that meet one or more of these technical criteria have been identified, and techniques to measure these biomarkers have been reported. In the following section we discuss the pros and cons of common biomarkers used to identify oxidative damage to biomolecules.

Biomarkers of Lipid Peroxidation

Lipids can be oxidized by numerous ROS and radicals. The resulting lipid peroxidation is a complex process that produces a variety of products (e.g., lipid hydroperoxides and aldehydes) in varying amounts (Halliwell & Whiteman, 2004). Lipid peroxidation can be measured in many ways, and each technique measures something different. A detailed discussion of each of these techniques is beyond the scope of this report. Nonetheless, here we discuss the pros and cons of some of the more common techniques currently in use.

Conjugated Dienes—ROS-mediated oxidation of polyunsaturated fatty acids forms conjugated dienes that absorb ultraviolet (UV) light at 230–235 nm. The ability to monitor conjugated dienes by spectrophotometric methods provides an assay that is both inexpensive and easy to perform. Therefore, the measurement of conjugated dienes has been used by many investigators as an index of lipid peroxidation. Although the assay of dienes is a useful index of the peroxidation of pure lipids and isolated lipoproteins, diene conjugation measurements in animal and human tissues or body fluids are problematic because many other substances present in the sample can absorb light in this UV range. Therefore, the validity of the measurement of conjugated dienes in tissues using UV techniques is questionable and the technique should be avoided (Halliwell & Whiteman, 2004).

Thiobarbituric Acid Assay—Another commonly used assay to assess lipid peroxidation in tissues is the thiobarbituric acid assay (TBA assay). The TBA assay is attractive because of its low cost and simplicity. In principle, this procedure is designed to measure the levels of malondialdehyde (MDA), a product of lipid peroxidation. The standard TBA assay is typically performed by heating the biological sample with TBA in an acid solution. During this process, TBA reacts with MDA to form a colored product (i.e., TBA-MDA adduct) that is extractable in organic solvents; this TBA-MDA adduct absorbs light at 532 nm and fluoresces at 553nm. The level of MDA in the sample is then determined by constructing a calibration curve using standard amounts of MDA. Unfortunately, there are numerous technical problems with this assay, and it has been argued that the standard TBA assay should be deemed unacceptable in modern research (Halliwell & Gutteridge, 2007; Halliwell & Whiteman, 2004). One of the major concerns with the standard TBA assay is that most of the TBA-reactive material in human body fluids is not related to MDA or lipid peroxidation. This concern alone renders this assay problematic, so the standard TBA assay is not recommended for measuring lipid-peroxidation products in human or animal tissues. For more details on the technical problems associated with the TBA assay, refer to reviews by Halliwell and colleagues (Halliwell & Gutteridge; Halliwell & Whiteman).

So, is the TBA assay useless? Many experts agree that the standard TBA assay should not be used in research, but high-pressure liquid chromatography (HPLC) assays can be used to

measure MDA, and these protocols eliminate much of the interference that plagues the standard TBA assay (Halliwell & Gutteridge, 2007). Note, however, that the HPLC-based TBA assay alone cannot be used to compare lipid peroxidation between tissues that differ in fatty-acid composition, because MDA originates from select fatty acids (Halliwell & Gutteridge).

Measurement of Total Lipid Hydroperoxides—Lipid peroxidation in both saturated and unsaturated fats results in the formation of highly reactive and unstable hydroperoxides. There are several techniques to measure lipid hydroperoxides in biological samples. A common approach is to measure hydroperoxides directly using redox reactions with ferrous ions (de Zwart, Meerman, Commandeur, & Vermeulen, 1999; Nourooz-Zadeh, 1999). The resulting ferric ions are then detected using the thiocyanate ion as the chromogen. This approach is relatively easy and inexpensive, although there are several experimental caveats. For example, ferric ions in the biological sample present a source of error (Halliwell & Gutteridge, 2007). Furthermore, many biological samples contain hydrogen peroxide and protein peroxides, which readily react with ferric ions to provide an overestimation of the total lipid hydroperoxides in the sample (Halliwell & Gutteridge). However, both of these problems can be circumvented by performing the assay in chloroform (Halliwell & Gutteridge, 2007). A final concern is that the reaction of lipid hydroperoxides with ferric ions will generate lipid radicals that could propagate peroxidation. Thus, the assay of total lipid hydroperoxides is not ideal for measuring lipid peroxidation in tissues.

Measurement of Aldehyde-Protein Conjugates—Numerous aldehydes are generated during lipid peroxidation, including 4-hydroxynonenal (4-HNE; Halliwell & Gutteridge, 2007). The assay of free aldehydes in biological samples is complicated, because the concentration of most aldehydes is low as a result of conjugation with cellular proteins (Halliwell & Gutteridge). Although several methods can liberate bound aldehydes, most techniques run the risk of artifactually increasing lipid peroxidation in the biological sample. In contrast, the determination of aldehyde-protein conjugates can be identified via antibody-based methods (e.g., Western blots or ELISA). The determination of 4-HNE-protein conjugates is reliable and can be used to identify specific proteins that are modified by conjugation with 4-HNE. Hence, the measure of 4-HNE-protein conjugates in tissues is a useful biomarker of oxidative damage in many experimental conditions.

F₂-Isoprostanes as a Marker of Lipid Peroxidation—Isoprostanes are important products of lipid peroxidation, and their measurement is currently the best assay to determine lipid peroxidation in biological samples. Isoprostanes are prostaglandin-like compounds formed from the oxidation of polyunsaturated fatty acids (PUFAs). Most of the work investigating the use of isoprostanes as a biomarker of oxidative stress has focused on the F₂-isoprostanes (Roberts & Morrow, 2002). Specifically, F₂-isoprostane (also called 8-iso-PGF_{2α}) is produced from the ROS-mediated arachidonic acid peroxidation independent of cyclo-oxygenase. Increased levels of F₂-isoprostanes are often observed in tissues exposed to oxidative stress. Moreover, F₂-isoprostanes have been detected in both plasma and urine in participants exposed to oxidative stress (Fam & Morrow, 2003; Milne, Sanchez, Musiek, & Morrow, 2007). The ability to detect F₂-isoprostane in urine provides a convenient and noninvasive index of in vivo oxidative damage. Although it has been suggested that F₂-isoprostane is a relatively stable molecule (Milne et al.), others report that free isoprostanes turn over rapidly and their half-life in human plasma is less than 20 min (Halliwell & Gutteridge, 2007). This relatively short half-life can be an important consideration for investigators when choosing sampling times in experiments.

Although isoprostanes can be detected in foods, only very limited amounts of these compounds can be absorbed, so diet has a limited effect on plasma or urine levels of isoprostanes (Halliwell

& Gutteridge, 2007). This is important because one of the criteria for evaluating markers of oxidative stress is that the biomarker should not be influenced by diet (Figure 1).

Sensitive and reliable gas chromatography/mass spectroscopy (GC-MS) assays have been developed to detect F₂-isoprostane, and there are also high-pressure liquid chromatography/gas spectroscopy (HPLC-GS) methods (Halliwell & Gutteridge, 2007; Milne et al., 2007). Although there are commercial immunoassay kits for measuring F₂-isoprostanes, the reliability of some of these methods has been questioned. For example, some authors report a strong correlations between GC-MS F₂-isoprostane values and values obtained by immunoassay (Basu, 1998; Sasaki et al., 2002), whereas others report significant but relatively weak correlations (i.e., $r = .62$, $p < .02$) between the two methods (Proudfoot et al., 1999). Therefore, future work is required to determine whether immunoassays for F₂-isoprostanes can reach the sensitivity and reliability of the well-established GC-MS methods.

Concluding Comments on Lipid-Peroxidation Biomarkers—From the preceding discussion, it can be concluded that none of the available techniques are ideal, but clearly some are better than others. Currently, it appears that measuring F₂-isoprostanes using GC-MS is an excellent assay of lipid peroxidation in biological samples. It is also possible that the assay of F₂-isoprostanes via immunoassay methods may also prove to be acceptable; however, more work is needed to confirm or deny this statement. Selected lipid-hydroperoxide assays and the determination of 4-HNE protein conjugates are also useful markers of lipid peroxidation in some experimental conditions. In contrast, determination of lipid peroxidation in tissues or body fluids using the standard TBA assay or conjugated dienes using UV methods is not recommended. When new methods for determining lipid peroxidation become available, investigators should evaluate the potential of each technique based on the technical criteria contained in Figure 1.

Biomarkers of Protein Oxidation

Proteins can be damaged by direct reaction with ROS, by reactions with end products of lipid peroxidation (e.g., 4-HNE), and by glycation. Exercise-induced oxidation of cellular proteins is of obvious importance because protein oxidation can impair the function of numerous proteins including enzymes, receptors, transport proteins, and contractile proteins. There are several techniques to detect oxidant-mediated protein damage in tissues, and each method detects something different (Dean, Fu, Stocker, & Davies, 1997). For a detailed discussion of methods used to measure oxidized proteins, the reader is referred to the classic free-radical biology text by Halliwell and Gutteridge (2007). Here, we will provide a brief overview of the strengths and weaknesses of four techniques that can be used to identify oxidatively modified proteins.

Carbonyl Assay—One of the most commonly used biomarkers of oxidatively modified proteins is the carbonyl assay that measures protein carbonyl groups in the biological sample. Note that although protein carbonyl formation can occur as a result of the direct oxidation of amino acid side chains by ROS, carbonyls can also be produced by protein glycation with sugars or by the binding of aldehydes to proteins (Halliwell & Whiteman, 2004).

The levels of protein carbonyls in biological samples can be determined spectrophotometrically or by immunochemical techniques. The assay of carbonyls using an immunochemical approach offers several advantages over the spectrophotometric method because this technique appears more reliable and has the advantage of being able to detect the levels of protein carbonyls in proteins differing in molecular weight (i.e., carbonyl groups identified in proteins separated via gel electrophoresis; Buss, Chan, Sluis, Domigan, & Winterbourn, 1997).

In synopsis, the carbonyl assay is a widely used technique to estimate the level of protein oxidation in biological samples. This technique is useful and provides a good measure of protein-oxidation levels in tissues. However, a few caveats about this technique warrant mention. First, in some cases of oxidative stress only a small number of proteins are oxidized in the biological sample (Halliwell & Gutteridge, 2007). Consequently, separating proteins via electrophoresis and measuring the levels of protein carbonyls in proteins that differ in molecular weight is a very useful means of evaluating oxidative damage to proteins in biological samples. In addition, it should be noted that oxidized proteins are recognized and degraded by the proteasome, so the measurement of protein carbonyls in a sample represents the flux between the generation of oxidized proteins and their removal (Halliwell & Whiteman, 2004). A final limitation of this technique is the high cost of reagent materials.

4-HNE-Protein Conjugates—As mentioned previously, the determination of 4-HNE-protein conjugates can be used to identify proteins that are oxidatively modified by conjugation with 4-HNE. Hence, the measure of 4-HNE-protein conjugates in tissues can be used as a marker of both lipid peroxidation and protein modification by oxidized products.

Assessment of Protein Thiol Oxidation—Many proteins contain thiol groups that can be oxidatively modified by ROS. Indeed, cysteinyl thiols can undergo a diverse array of redox reactions that are largely dependent on the species and concentrations of oxidants present in the cell (Eaton, 2006). Although not all protein thiols interact with oxidants at the concentrations found in cells, some protein thiols do react with ROS, forming protein disulfides and so lowering the total number of thiol groups present in proteins contained in a biological sample (Eaton). Thus, some investigators have measured total protein thiols in biological samples as a marker of exercise-induced oxidative modification of muscle proteins (McArdle, Pattwell, Vasilaki, Griffiths, & Jackson, 2001; Vincent, Powers, Demirel, Coombes, & Naito, 1999). Many methods are available for measuring the reduced protein thiol status in tissues, but most use chemicals that react with thiol groups to form a label that can be detected. Several of these techniques appear reliable and are relatively easy to perform and inexpensive. Nonetheless, because protein thiols in biological samples can be modified during tissue thawing and the homogenizing process, special care is required to avoid this problem.

Analysis of Oxidized Amino Acids—Oxidatively modified amino acids (e.g., ditryptosine) have been used as a biomarker of exercise-induced oxidized proteins in skeletal muscle (DiMarco & Giulivi, 2007; Leeuwenburgh, Hansen, Holloszy, & Heinecke, 1999). The measurement technique uses GC-MS to measure trace amounts of unnatural oxidized amino acids in tissues and can identify both the magnitude of protein oxidation and the source of oxidant-mediated protein damage in the sample (Leeuwenburgh, Hansen, Shaish, Holloszy, & Heinecke, 1998; Leeuwenburgh et al., 1999).

Although the GC-MS approach to measuring oxidized amino acids appears to be sensitive and reliable, there are two significant disadvantages of this approach. First, proteins can be oxidized during cooking and these oxidatively modified amino acids can be absorbed into the body. This results in a confounding variable in studies of exercise-induced oxidative stress (Halliwell & Gutteridge, 2007). Second, this technique is complex, time consuming, and expensive. Thus, only a small number of research groups have used the technique.

Concluding Comments on Protein-Oxidation Biomarkers—Similar to our discussion on biomarkers of lipid peroxidation, none of the available biomarkers of protein oxidation is ideal, but some biomarkers are superior to others. At present, the detection of protein carbonyls using immunoblotting techniques is considered to be one of the best biomarkers of protein oxidation in biological samples. Careful application of selected protein thiol assays and the determination of 4-HNE protein conjugates are also useful markers of protein modification in

select experiments. When novel techniques for measuring oxidized proteins become available, scientists must evaluate the promise of each protocol based on the principles presented in Figure 1.

Biomarkers of DNA Damage

Oxidative damage to DNA followed by repair appears to occur continuously in cells. DNA subjected to attack by different radical species generates a large range of base- and sugar-modification products, and many of these products have been used as biomarkers of oxidative damage to DNA. In general, measurements of DNA damage can be classified into two broad categories. The first measures DNA damage in cells; this assessment reflects the balance between DNA damage and repair and therefore represents steady-state damage. It follows that a rise in the level of damaged DNA in cells could occur because of increased DNA damage, decreased repair, or some combination of the two.

The second category of DNA-damage measurements is the estimate of total oxidative DNA damage and involves the detection of damage products excreted in the urine. Several damaged DNA bases can be excreted in the urine and measured by a variety of methods. However, because urine contains thousands of compounds, proper sample preparation is critical for these measures (Halliwell & Gutteridge, 2007). Some authors question the validity of measuring damaged DNA bases in the urine because many of damaged DNA bases can be derived from the diet (e.g., DNA damaged by cooking; Halliwell & Gutteridge).

Several approaches have been used to evaluate DNA damage in exercise studies (Neubauer, Reichhold, Nersesyan, Konig, & Wagner, 2008; Poulsen, Weimann, & Loft, 1999). A detailed discussion of all of these techniques is beyond the scope of this review. Nonetheless, in the following segments we provide an overview of the two most commonly used methods to detect DNA damage in exercise studies.

8-Hydroxyl-2'-Deoxyguanosine—Most attempts to “fingerprint” oxidative damage to DNA have measured modified bases, with the most frequently used single marker of oxidative DNA damage being 8-hydroxyl-2'-deoxyguanosine (8OHdG). After DNA isolation, 8OHdG can be measured using numerous approaches including HPLC, GC-MS, and antibody-based techniques (Halliwell & Gutteridge, 2007).

It has been argued that measuring 8OHdG from DNA isolated from tissues is problematic because 8OHdG can be formed during isolation of DNA (Gedik & Collins, 2005; Halliwell & Whiteman, 2004). One approach to bypass this problem is to measure the DNA damage within intact cells. In this regard, there are antibody techniques to measure 8OHdG that are useful for visualizing DNA damage in cells, but they are semiquantitative measures. Because of the methodological drawbacks associated with this biomarker of DNA damage, some authors have urged caution in the interpretation of data using this biomarker as the single measure of DNA damage (Knasmuller et al., 2008; Neubauer et al., 2008).

Single-Cell Gel Electrophoresis (i.e., Comet Assay)—Another approach that bypasses the problems associated with DNA isolation involves measuring DNA damage in cells using the single-cell gel electrophoresis assay. This assay is based on the determination of DNA migration in an electric field, which leads to the formation of comet-shaped images; hence, this assay is commonly referred to the comet assay. The comet assay can be applied directly to cells and measures DNA strand breaks. This technique appears to be a valid measure of DNA damage in vivo, but a number of criteria must be met in order for the technique to produce reliable results (Gedik, Boyle, Wood, Vaughan, & Collins, 2002; Hartmann et al., 2003; Knasmuller et al., 2008).

For exercise studies, a disadvantage of the comet assay is that the technique cannot be applied to bundles of muscle fibers obtained via muscle biopsy. Nonetheless, this procedure can and has been used to determine whether antioxidant supplementation protects leukocytes against exercise-induced DNA damage (Mastaloudis et al., 2004). Furthermore, this assay can be used to measure DNA strand breaks in cultured myotubes exposed to oxidant stress.

Concluding Comments on DNA-Damage Biomarkers—Because of the invasive nature of obtaining human muscle samples, most studies investigating exercise-induced DNA damage have examined DNA damage in white blood cells (often lymphocytes) or damaged DNA products excreted in the urine. However, when designing experiments to investigate exercise-induced DNA damage, investigators should consider two points. First, there is no evidence that DNA damage in white blood cells accurately reflects the level of DNA damage in other tissues including skeletal muscles. Second, although measurement of DNA damage products in the urine may reflect the total DNA damage in the body, this measurement does not indicate the cellular source of DNA damage.

In summary, although there are numerous techniques to measure DNA damage, recent reviews on this topic have concluded that none of these techniques represents a true “gold standard” (Halliwell & Whiteman, 2004; Knasmüller et al., 2008). Thus, it has been suggested that studies should include two or more different measures of DNA damage (Halliwell & Whiteman). Furthermore, the best measurement of DNA damage would include measurements of both cellular DNA damage and total DNA damage via urinary excretion rates (Halliwell & Gutteridge, 2007).

Biomarkers of Redox Disturbances in Tissues

In the absence of oxidative stress, cells maintain a relatively stable redox state that is primarily determined by thiol systems comprising three major redox couples: glutathione, thioredoxin, and the cysteine/cystine couples (Kemp, Go, & Jones, 2008). It is interesting that these redox couples are not in equilibrium with each other, and each is strategically located within the mitochondrial, nuclear, and cytosolic compartments of the cell (Go, Pohl, & Jones, 2009; Kemp et al.).

Because of the high cellular concentrations, the glutathione/glutathione-disulfide couple is a major contributor to the cellular redox state (Jones, 2008). Although both the thioredoxin and cysteine/cystine redox couples contribute to cellular redox control, the concentrations of these couples are much lower than that of glutathione and therefore contribute less to the total cellular redox state (Go et al., 2009). Hence, because cells contain glutathione in relatively high levels (1–10 mM), many studies have measured changes in the glutathione redox system as a marker of cellular redox status. For example, during periods of oxidative stress, the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is lowered in the cell, resulting in a disruption of the normally high ratio (i.e., >100/1) of GSH to GSSH. Therefore, researchers have often used changes in the plasma or muscle-tissue GSH:GSSH ratio as a marker of oxidative stress. However, alterations in the GSH:GSSH ratio reflect changes in the cellular redox state and are not a direct biomarker of oxidative damage because the cellular GSH:GSSH ratio can decrease without the appearance of oxidized lipids or proteins (Halliwell & Gutteridge, 2007). Moreover, the measurement of plasma or muscle GSH:GSSH requires great care because GSH is easily oxidized during both isolation and the assay (Halliwell & Gutteridge).

The measurement of changes in cellular redox couples (i.e., GSH:GSSH) has been incorporated into many exercise studies as a marker of exercise-induced redox disturbances. When measured carefully using appropriate safeguards to protect against artifactual GSH oxidation,

measurements of the GSH:GSSH ratio in plasma and tissues are useful when combined with additional measures of oxidative biomarkers.

Commentary on Interpretation of Biomarker Results

Several comments regarding the interpretation of measurements of oxidative damage to cellular components are appropriate. First, it is important to understand that all biomarkers of oxidative damage to tissues are subject to turnover, so the level of a specific biomarker does not reflect the total amount of oxidative damage at a given time point. Therefore, it is improper to discuss group differences in a specific biomarker as a percentage difference in oxidative damage. In contrast, it is appropriate to state that groups are statistically different in the levels of a specific biomarker (e.g., lipid peroxidation) by a “certain percent” because this reflects the actual measurement being quantified.

Because of the invasive nature of a muscle biopsy, many human antioxidant studies have measured exercise-induced changes in biomarkers of oxidative damage detected in venous blood samples. In many cases, authors have assumed that an increase in oxidized biomarkers found in the blood is a direct result of increased radical production in the active skeletal muscles. Although this may be the case, it is not an experimentally demonstrated truth. In fact, it is feasible that other tissues such as the heart, lungs, or white blood cells may contribute significantly to the total body generation of ROS in some situations (Powers & Jackson, 2008). Therefore, researchers should use caution when extrapolating findings from blood samples to events occurring in other tissues.

Is There a Single Biomarker of Oxidative Damage?

The short answer is no, although several biomarkers of oxidative damage appear to be sensitive and reliable and rise in parallel in cells subjected to oxidative stress (Halliwell & Gutteridge, 2007). Nonetheless, as discussed previously, none of the available biomarkers of oxidative stress are ideal, but some are better than others. Furthermore, the time course of biomarker formation and removal differs between various biomarkers. Therefore, when planning a rigorous study, it is recommended that investigators consider including two or more of the “acceptable” biomarkers of cellular oxidative damage in their experiments. For example, the finding that three different biomarkers of oxidative stress increase in parallel during an exercise session, and all three markers are depressed during the exercise performed with participants consuming an antioxidant supplement, provides strong evidence that the test supplements are effective in protecting against exercise-induced oxidative damage to the specific tissues analyzed. A brief summary of oxidative-stress biomarkers is presented in Table 1.

Antioxidant-Capacity Assays

In designing experiments to investigate the effect of antioxidant supplementation on human performance it is valuable to determine whether the antioxidant treatment results in a significant increase in the total antioxidant capacity of blood and other tissues (e.g., skeletal muscle). In this regard, numerous methods have been developed to assess the total antioxidant capacity of foods, beverages, and body tissues. A global assay that measures the total antioxidant capacity is advantageous because it is both expensive and time consuming to measure each individual antioxidant component in a tissue. Moreover, the possibility of cooperative interactions among individual antioxidants makes measuring the total antioxidant capacity appealing. Unfortunately, there is not currently a single assay that can reliably and validly measure total antioxidant capacity in tissues. Nonetheless, several techniques to assay total antioxidant capacity are available, and in the following section we will discuss the pros and cons of three of the most common methods in use today.

Oxygen-Radical Absorbance-Capacity Assay

The oxygen-radical absorbance-capacity (ORAC) assay measures the ability of antioxidants in a sample to protect a protein from oxidative damage by peroxy radicals. A loss of conformation occurs as the protein suffers oxidative damage, resulting in a decrease in fluorescence (Cao, Alessio, & Cutler, 1993). Initially, the protein of choice for the ORAC assay was β -phycoerythrin (β -PE). However, using β -PE in the ORAC assay presents several problems including inconsistency between batches, interaction with phenolic compounds leading to nonspecific protein binding, and variability because of photosensitivity (Cao & Prior, 1999; Ou, Hampsch-Woodill, & Prior, 2001). An improved method of the ORAC assay uses fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) as the fluorescent probe. Compared with β -PE, fluorescein is less expensive, does not interact with other compounds, and is very stable. However, when the pH drops below 7.0, the intensity of fluorescein decreases drastically (Ou et al.). Other downsides of the ORAC assay include the need for expensive equipment, variability across instruments, and lengthy analysis time (Zulueta, Esteve, & Frigola, 2009). Furthermore, the assay is performed in aqueous solution and therefore primarily measures hydrophilic antioxidant activity. Nonetheless, the determination of antioxidant capacity using the fluorescein-based ORAC assay is effective in many experimental models and conditions, so it is currently one of the best assays of antioxidant capacity.

Trolox-Equivalent Antioxidant-Capacity Assay

The trolox-equivalent antioxidant-capacity (TEAC) assay is based on the scavenging of a radical by antioxidants present in a sample. During the TEAC assay a sample of biological material is added to a free-radical-generating system, and inhibition of the free-radical reaction is proportional to the antioxidant capacity of the material (Cao & Prior, 1998). The TEAC assay is based on the inhibition of the ABTS radical (generated by 2,2'-azino[3-ethylbenzothiazoline-6-sulfonate]) by antioxidants present in the sample. The ABTS radical typically has a maximum absorbance value of 734 nm. The antioxidants quench the ABTS radical, resulting in a loss of color and thus a decrease in absorbance (Re et al., 1999). The change in absorbance quantitatively corresponds to the concentration of antioxidants present in the system. The TEAC assay is inexpensive, fast, easy to perform, and stable to changes in pH. However, it has several limitations. For example, the ABTS radical is relatively unstable. In addition, there are different TEAC-assay methods, making it difficult to compare TEAC results from studies using different variations of the assay. Furthermore, unlike the ORAC assay, the TEAC assay is based on a one-electron transfer and so cannot measure chain-breaking antioxidant activity. Hence, some authors have concluded that the TEAC assay has limited validity in measuring total antioxidant capacity and should be used with caution (Frankel & Meyer, 2000).

Ferric-Ion-Reducing Antioxidant-Power Assay

The ferric-ion-reducing antioxidant-power (FRAP) assay is based on reducing agents (antioxidants) reducing the ferric tripyridyltriazine complex (Fe^{3+} -TPTZ) to the ferrous complex (Fe^{2+} -TPTZ) at low pH (~3.4). Like the TEAC assay, the FRAP assay is inexpensive and easy to perform and thus has been commonly used in research to evaluate in vitro antioxidant capacity. Nonetheless, there are several problems with the FRAP assay. Like the TEAC assay, it is based on a single electron transfer and so cannot measure the total capacity of antioxidants such as chain-breaking antioxidant activity. Furthermore, although the redox potential of Fe^{3+} -TPTZ (~0.70 V) in the FRAP assay is comparable to that of ABTS• (~0.68 V) used in the TEAC assay, the FRAP assay is carried out at a very low and nonphysiological pH of 3.4, compared with the neutral pH used in the TEAC assay (Huang, Ou, & Prior, 2005). In addition, the absorption (A_{593}) of some antioxidant polyphenols such as caffeic acid,

tannic acid, ferulic acid, ascorbic acid, and quercetin does not stop during the 4-min reaction time of the FRAP assay, instead, slowly increasing for several hours (Pulido, Bravo, & Saura-Calixto, 2000). Thus, when analyzing samples containing polyphenols, the 4-min reaction time of the FRAP assay is inadequate and does not measure the true antioxidant capacity. Furthermore, the FRAP assay does not detect the activity of important thiol antioxidants such as glutathione. Therefore, because of these multiple drawbacks, the FRAP antioxidant assay is less than ideal.

Concluding Comments on Antioxidant-Capacity Assays

Although there is no single assay that is capable of reliably and validly measuring total antioxidant capacity, the ORAC assay using fluorescein appears to be one of the best single antioxidant-capacity assays currently available. Nevertheless, to comprehensively measure total antioxidant capacity, validated and specific assays are needed in addition to ORAC. Indeed, a total antioxidant-capacity assay that measures the antioxidant capacity against one ROS species is not biologically realistic (Huang et al., 2005). Therefore, when assessing total antioxidant capacity of tissues, it is prudent to evaluate the antioxidant potential of tissues after exposure to a wide variety of biologically relevant ROS (Coombes et al., 2000). A summary of the major pros and cons of antioxidant-capacity assays is presented in Table 2.

General Conclusions

From the preceding discussion, it should be clear that designing a rigorous experiment to investigate the effect of antioxidant supplements on human performance is complex and requires careful attention to detail. A robust experimental design includes careful selection of antioxidants for study, suitable antioxidant dosage, the appropriate duration of antioxidant supplementation, and attention to dietary control. Regardless of the antioxidant compounds selected for study, each compound should be analyzed to ensure purity. Moreover, well-designed experiments will also assess the bioavailability of the antioxidants in the plasma or skeletal muscle of the experimental participants. Selection of a laboratory exercise test that is valid, reliable, and sensitive is essential for any study designed to investigate the influence of a nutritional intervention on human performance. Furthermore, all antioxidant experiments should include two or more acceptable biomarkers of oxidative damage in tissues. Finally, a detailed experimental plan will also include assays to determine the antioxidant capacity of tissues (e.g., blood or muscle tissue) as an index of whether the antioxidant treatment was successful in increasing the oxidant-scavenging capacity of the tissues.

Acknowledgments

This work was supported by NIH R01HL087839.

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- The biomarker should detect a fixed percentage of the total ongoing oxidative damage in vivo.
- The coefficient of variation between assays should be small (ideally, <10%).
- Measurement of the biomarker should employ chemically robust techniques.
- The level of the biomarker in vivo must not be influenced by diet.
- The biomarker should be stable during storage (i.e., the biomarker should not be lost or formed by artifact during storage).

Figure 1.

Technical criteria for the ideal biomarker of oxidative damage in tissues. Modified from Halliwell and Whiteman (2004).

Table 1

Summary of Oxidative-Stress Biomarkers

Assay	Pros	Cons
Conjugated dienes	Inexpensive, easy to perform	Low validity
Thiobarbituric acid	Inexpensive, simple	Numerous technical problems
Lipid hydroperoxides	Inexpensive	High error, commonly overestimates
4-HNE	Reliable	Risk of artifact, somewhat complicated
F2-isoprostanes	Reliable, sensitive (GC-MS)	Expensive, difficult (GC-MS)
Protein carbonyls	Reliable, specific (electrophoresis)	Expensive
Protein thiol oxidation	Inexpensive, easy to perform	Risk of artifact
Oxidized amino acids	Sensitive, reliable (GC-MS)	Complex, time consuming, expensive
8OhdG	Numerous available assays	High risk of artifact, semiquantitative
Comet assay	Valid (in vivo)	Difficult, cannot use biopsied muscle

Table 2

Summary of Antioxidant-Capacity Assays

Assay	Pros	Cons
Oxygen-radical absorbance capacity	Sensitive	Expensive, time consuming, variability across instruments
Trolox-equivalent antioxidant capacity	Inexpensive, quick	Lacks validity because of multiple limitations
Ferric-ion-reducing antioxidant power	Inexpensive, easy	Lacks validity because of multiple limitations