Evaluation of antioxidants effects from grape seed extract consumption based on blood analysis of series of indicators on 110 random persons

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Summary

In recent years, increasing attention has been paid to functional foods with antioxidative effects. Hence, comprehensive and precise evaluation of the effects of antioxidant food has also been a focus of food research. This study aimed to evaluate the validity and rationality of antioxidant indices and methods in vivo. One hundred and ten participants were randomly allocated to receive standardized grape seeds or placebo capsules. After 90 days of using either antioxidant preparations or placebo, antioxidant effects were measured based on the serum levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), malondialdehyde, 8-isoprostaglandin and 3-nitrotyrosine. An antioxidant capability was considered if any two of the first three indices were positive, which means that self-comparison and between-groups levels significantly changed after 3 months. The results showed that the serum levels of all the tested antioxidant indices improved significantly ($P < 0.01$) after the use of grape seed extract. More importantly, the differences in SOD and GSH-PX levels were significant between the groups. In conclusion, SOD as well as GSH-PX levels can be effective indices and the method we utilized can provide helpful references for evaluation standards of the antioxidant function.

Keywords

bioactivity; antioxidant function; grade seeds; superoxide dismutase; glutathione peroxidase

It is generally believed that one of the main causes of ageing is peroxidation of cells or tissues caused by free radicals and other harmful factors in the process of metabolism [1]. Under normal conditions, human body produces free radicals when it reacts with oxygen, while production and elimination of free radicals are dynamic. When external conditions destroy the balance between free radical production and elimination, biological macromolecules in the body are destroyed, the function of tissues and organs is affected, and the ageing speed is accelerated, leading to a series of diseases, including cancer [2], atherosclerosis [3] and diabetes mellitus [4]. Therefore, increasing attention has been paid to antioxidants. The term antioxidant refers to any chemical compound with the ability to either stabilize or downgrade free radicals, fight against the increasing oxidative stress, reduce cumulative oxidative damage and protect the body from damage caused by free radicals [5]. Under conditions of increased oxidative stress, endogenous antioxidants need not be sufficient, requiring supplementation with exogenous antioxidants in the daily diet [6].

The interest in finding food with antioxidant function to ensure a diet that maintains free radical balance has increased considerably over the past years. However, many evaluative indices exist, which are ambiguous or complicated. The evaluation methods for antioxidative effects also need to be further improved. Therefore, we carried out a placebo-controlled, randomized,

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double-blind clinical trial to evaluate the antioxidant function of standardized grape seeds. Proanthocyanidins, which belong to the most effective components in grape seeds, have strong antioxidant activity and are good free radical scavengers as well as lipid peroxidation inhibitors. BAGCHI et al. [7] found that proanthocyanidins have high bioavailability and a significantly higher ability to resist free radicals as well as to protect DNA against damage compared to vitamin C, vitamin E and β-carotene. Proanthocyanidins have been applied in medicine, health products, food technology and other fields [8]. Therefore, we use grape seed compounds, including abundant proanthocyanidins, that have been approved to be marketed and recognized as health-promoting foods with antioxidant function, to verify the feasibility of the current indicators commonly used in evaluation of antioxidant function and to explore new indicators.

In the human body, a variety of unique oxidation-reduction reactions takes place. Antioxidant food induces a series of complex interactions. Therefore, in addition to animal experiments, it is necessary to carry out clinical trials for their verification. Currently, there are dozens to hundreds of evaluation methods for antioxidant functional foods. Although these are complex, they can be divided into three categories: in vitro antioxidant, cell antioxidant and in vivo antioxidant methods [9, 10]. The in vivo antioxidant approach can better simulate the complex biological environment in the human body, leading to evaluative results with a stronger biological correlation. Therefore, we chose the in vivo antioxidant method to measure antioxidant effects.

Reactive oxygen species (ROS) oxidize various biological macromolecules, such as proteins, lipids or nucleic acids, thereby causing changes in the structure and function of these molecules. As such, the oxidation state can be evaluated accordingly. The reaction of ROS with lipids is known as “lipid peroxidation” and its widely accepted biomarkers include malondialdehyde (MDA) and 8-isoprostaglandin [11]. Modification of proteins and nucleic acids can also be used to measure the oxidative damage and 3-nitrotyrosine indicates the oxidative damage of proteins [12]. In addition, the ability of the body to resist oxidation can be detected by measuring the levels and activity changes of antioxidant enzymes and antioxidants, such as superoxide dismutase (SOD) or glutathione peroxidase (GSH-PX) [13, 14]. SOD removes superoxide radicals in the body [15], while GSH-PX can remove substances such as H_2O_2 or organic hydroperoxides [16].

We selected the five aforementioned indicators to measure the antioxidant effect and evaluated the reliability of the said indicators in measuring food antioxidant function to provide suggestions for the future standardization and revision of the evaluative indices and methods of the antioxidant function in vivo.

MATERIALS AND METHODS

Study design

A randomized, double-blind, placebo-controlled study with a parallel design was performed at the Xiyuan Hospital of China Academy of Chinese Medical Sciences (Beijing, China). In accordance with the Declaration of Helsinki, all patients provided written, informed and voluntary consent to participate.

According to the provisions of relevant national departments, considering 20 % of the shedding factors, 120 patients were observed, with 60 cases in the test group and 60 cases in the placebo group. Patients were eligible for enrolment if they were 18–65 years old, in good health, without obvious brain, heart, liver, lung, kidney or blood diseases and had no long-term medication history. The following patients were excluded from enrolment: (1) pregnant or lactating women, (2) allergic to a variety of drugs or known to be allergic to the components of this product such as proanthocyanidins, grape seed extracts and bilberry extracts, (3) had heart, liver, kidney or hematopoietic system diseases, (4) did not take the test product according to the regulations, especially incorrect dosage or insufficient course of treatment, and (5) consumed other health-promoting foods or drugs that could affected the test outcome. Ultimately, ten patients were removed. For the research and statistical analyses, we used the data of 110 participants who managed to consume the antioxidant preparations or placebos in the prescribed way for the period of the study. There were no significant differences in age, sex, antioxidant index or symptom scores between the two groups.

Detection of efficacy indicators included the serum levels of malondialdehyde (MDA), superoxide dismutase (SOD, EC 1.15.1.1), glutathione peroxidase (GSH-PX, EC 1.11.1.9), 8-isoprostaglandin and 3-nitrotyrosine (3-NT). If changes in the MDA, SOD, and GSH-Px levels were statistically significant, be it by self-comparison or between-group comparison, the indicator was considered positive. If any two indicators of MDA, SOD and GSH-Px levels were positive, we concluded that the sample had an antioxidant function.

Antioxidant preparations and biochemical measurements

All participants were randomly allocated to receive standardized grape seed extract or placebo capsules (Xianle Health Technology, Guangdong, China). The participants were instructed to consume two grape seed capsules once a day, with one capsule containing 92.5 mg of standardized dry extract of proanthocyanidin. The placebo capsules were made from microcrystalline cellulose, a material that does not affect the antioxidant level. The patients were not allowed to consume health-promoting foods or medicines with the same efficacy during the trial. The treatment course lasted for 90 days and the results were observed after.

Morning fasting vein blood samples were used to evaluate the MDA, SOD, GSH-Px, 8-isoprostaglandin and 3-NT levels. Serum was separated and stored at –20 °C for a maximum of 4 h. All biochemical assessments were performed in the same laboratory using standard methods.

The 8-isoprostaglandin level was measured with an 8-isoprostaglandin ELISA Kit (Cayman, Ann Arbor, Michigan, USA) according to the manufacturer's instructions and using its own kits. The 3-NT levels were measured using a 3-NT ELISA Kit (MIBio, Shanghai, China) according to the manufacturer's instructions and using its own kits. The SOD levels were measured using a Microlab 300 semi-automatic biochemical analyser (Vital Scientific, Spankeren, The Netherlands) according to the instructions provided by Randox Laboratories (Crumlin, United Kingdom) and using its own kits.

Symptom score

The symptom score was used to assess the patient's quality of life and response to the antioxidant function of health-promoting foods. It examines four clinical symptoms of fatigue, irritability, sleep and soreness in the waist and knees. Each symptom was evaluated and categorized on a scale from 0 to 3, leading to a maximum score of 12 points. The numerals denoted the following: 0 = no reaction, 1 = mild reaction, 2 = moderate reaction, and 3 = strong reaction.

Statistical analysis

Analysis was performed using the SPSS software (version 19.0; IBM, Armonk, New York, USA). The values were presented as mean ± standard deviation. Differences between means in groups were calculated using a two-sample *t*-test. The paired sample t-test was used for the between-group analysis. The limit of significance was defined as a two-sided *p*-value of < 0.05.

RESULTS AND DISCUSSION

In our study, 110 participants were randomly allocated into two groups. Of the 110 participants, 13 (11.8 %) were male and 97 (88.2 %) were female. Before the trial, no significant differences were found between the groups. The results of the in-group and between-group comparisons of the baseline characteristics are shown in Tab. 1. At baseline, the between-group analysis showed no significant differences in the serum levels of SOD, GSH-PX, MDA, age and sex. All the antioxidant indices of the test group showed significant differences after the intake of the antioxidant preparations (Tab. 2). Serum levels of SOD and GSH-PX significantly increased after a 90-day consumption of the preparation.

In the placebo group, only the serum levels of 8-isoprostaglandin and 3-NT showed significant differences after the trial (Tab. 3). Between-group analysis showed that the differences in the serum levels of SOD and GSH-PX were significant. No statistically significant differences were detected in the MDA, 8-isoprostaglandin and 3-NT levels (Tab. 4).

In this study, we found that consuming this health-promoting food can significantly improve the serum levels of SOD and GSH-Px in vivo. Also, it further confirmed the indices' reliability in measuring food antioxidant function. In addi-

Tab. 1. Differences in participants who used grape seed extract and placebo at baseline.

	Participants who used grape seed (n = 55)	Participants who used placebo (n = 55)	P
Age years	57.56 ± 6.07	56.51 ± 5.08	0.325
Gender	5 males, 50 females	8 males, 47 females	0.376
Superoxide dismutase (SOD) [U·ml^{-1}]	115.73 ± 16.99	116.55 ± 17.50	0.805
Glutathione peroxidase (GSH-PX) [U·ml^{-1}]	154.68 ± 16.65	155.40 ± 12.72	0.801
Malondialdehyde (MDA) [nmol·ml^{-1}]	5.34 ± 1.35	5.73 ± 1.72	0.214
Symptom score	2.49 ± 1.88	2.40 ± 2.23	0.818

Tab. 2. Differences in antioxidant indices in participants who used grape seed extract after trial.

	Differences	*T*	*P*
Superoxide dismutase (SOD) [U·ml^{-1}]	8.74 ± 15. 34	–4.228	0.000
Glutathione peroxidase (GSH-PX) [U·ml^{-1}]	9.78 ± 16.42	–4.418	0.000
Malondialdehyde (MDA) [nmol·ml^{-1}]	–0.50 ± 1.79	2.068	0.043
8-Isoprostaglandin [nmol·ml^{-1}]	110.51 ± 31.09	3.550	0.001
3-nitrotyrosine (3-NT) [nmol·ml^{-1}]	0.56 ± 0.11	5.259	0.000

T-statistic is the difference between the average values of antioxidant indices in the group.

Tab. 3. Differences in antioxidant indices in participants who used placebo after trial.

	Differences	*T*	*P*
Superoxide dismutase (SOD) [U·ml^{-1}]	2.56 ± 16.35	–1.161	0.251
Glutathione peroxidase (GSH-PX) [U·ml^{-1}]	3.06 ± 13.84	–1.638	0.107
Malondialdehyde (MDA) [nmol·ml^{-1}]	–0.30 ± 2.97	0.746	0.459
8-Isoprostaglandin [nmol·ml^{-1}]	63.99 ± 15.56	4.110	0.000
3-nitrotyrosine (3-NT) [nmol·ml^{-1}]	0.65 ± 0.12	5.455	0.000

T-statistic is the difference between the average values of antioxidant indices in the group.

Tab. 4. Differences in antioxidant indices in participants between groups after trial.

	Differences in participants who used grape seed (n = 55)	Differences in participants who used placebo (n = 55)	*T*	*P*
Superoxide dismutase (SOD) [U·ml^{-1}]	8.74 ± 15. 34	2.56 ± 16.35	2.046	0.043
Glutathione peroxidase (GSH-PX) [U·ml^{-1}]	9.78 ± 16.42	3.06 ± 13.84	2.323	0.022
Malondialdehyde (MDA) [nmol·ml^{-1}]	–0.50 ± 1.79	–0.30 ± 2.97	–0.429	0.669
8-Isoprostaglandin [nmol·ml^{-1}]	110.51 ± 31.09	63.99 ± 15.56	–1.338	0.186
3-nitrotyrosine (3-NT) [nmol·ml^{-1}]	0.56 ± 0.11	0.65 ± 0.12	0.542	0.591

T-statistic is the difference between the average values of antioxidant indices between the two groups.

tion, the symptoms of fatigue and back aching were effectively relieved, implying that the improvement of clinical symptoms may be included in the evaluative standards of antioxidant function. However, statistically significant differences in the serum levels of MDA, 8-epihydroprostaglandin and 3-NT were not found in this study. In future studies, the sample size can be increased.

Ageing is a complicated and inevitable physiological phenomenon in life. One of the most representative ageing theories is the free radical theory proposed by HARMAN in 1956 [1, 17, 18]. According to this theory, free radicals with strong bioactivities damage tissues and cells. As the body ages, mitochondrial function gradually loses its ability to meet the demands of oxidative phosphorylation, which consequently results in levels of excessive free radicals and, in turn, causes more damage to mitochondrial function. When the cell function can no longer be maintained, the tissues and organs begin to degenerate, further aggravating the ageing state [19, 20].

Human body has complex antioxidant systems (enzymatic and non-enzymatic) that work synergistically to protect the body's cells and organ systems against free radical damage. However, with increasing age, the synthesis and activity of antioxidant enzymes decrease, and the body's ability to scavenge free radicals decreases, resulting in an imbalance in the production and removal of free radicals. An increasing number of studies confirmed that enhancing the body's antioxidant and free radical-scavenging capacities can delay ageing and prevent various diseases. Hence, the research on and exploration of antioxidant foods, tohether with appropriate evaluative standards to measure their antioxidant effect, have become a key topic in the field of food and medicine.

The imbalance between ROS production and enzymatic or non-enzymatic antioxidants can result in oxidative stress [21]. Biomarkers of oxidative stress are relevant in the evaluation of the health-enhancing effects of antioxidants. However, there is a lack of standardization in

the methods used for measuring antioxidant capacity in the human body. Under certain conditions, such as exposure to damaging agents or restricted capabilities of endogenous antioxidant systems, ROS are produced [22]. Of these, the most common agents are the reactive oxygen and nitrogen species (ROS/RNS), which are produced under oxidative stress and subsequently damage all cellular biomolecules (lipids, sugars, proteins and polynucleotides) [23, 24]. Thus, several defence systems are present within cells to prevent uncontrolled ROS increase. These systems include non-enzymatic molecules (glutathione; vitamins A, C and E, together with several antioxidants present in foods) and enzymatic scavengers of ROS, with SOD, catalase (CAT) and GSH-PX being the best-known defence systems [21]. Therefore, some studies evaluated the antioxidant indicators used to measure evaluate the effects of antioxidant supplements: (1) ROS in leukocytes and platelets by flow cytometry, (2) biomarkers resulting from ROS-induced modifications of lipids, DNA and proteins, and (3) enzymatic roles of the redox status [22].

Direct quantification of ROS/RNS is a valuable and promising biomarker that can reflect the antioxidant levels in the human body. However, the short half-life of these species increases the difficulty of their measurement in biological systems. Nonetheless, with a limitation to cell cultures and other in vitro applications, some methods, including electron spin resonance, fluorescence magnetic resonance and mass spectrometry techniques, can measure these species to some extent [24, 25]. Flow cytometry is one of the most powerful tools for the single-cell analysis of antioxidant systems. Many fluorescent probes for the detection of reactive species have been developed in recent years, with different degrees of specificity and sensitivity. With functions in detecting different origins of reactive species, dihydrochlorofluorescein diacetate, 4,5-diaminofluorescein diacetate, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate and dihydrorhodamine 123 (DHR123) as fluorescent probes have widely been used for ROS/RNS detection in blood cells via flow cytometry [26–28]. On the other hand, in addition to measurement of free radical production, it is also effective to measure some stable markers that may reflect a systemic or tissue-specific oxidative stress. Lipids, DNA and proteins are examples of molecules that can be modified by interactions with excessive ROS in vivo [29].

Regarding lipids, polyunsaturated fatty acids, mainly arachidonic acid, are highly susceptible to oxidative damage in the presence of ROS or free radicals [30]. MDA and 4-hydroxy-2-nonenal, the most investigated end-products of lipid oxidation, are the widely used markers of oxidative stress [31]. Oxidation of DNA components due to interaction with ROS/RNS can generate various products, with 7,8-dihydroxy-8-oxo-2-deoxyguanosine being the most common, which is also the most commonly used biomarker of DNA oxidation to measure oxidative stress [32]. DNA damage may also be caused by the attack of reactive products resulting from the ROS-induced modifications of other molecules, such as lipids. In this case, the etheno-DNA adducts, such as 1,*N*(6)-etheno-2-deoxyadenosine and 3,*N*(4)-etheno-2-deoxycytidine, are produced and can be used as biomarkers of oxidative stress [33].

Proteins represent a broad target for ROS and RNS generated under normal or oxidative stress conditions and can be considered as general scavengers of these species. The measure of carbonyl levels in proteins is the most widely used marker of oxidative protein damage, and tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins [34]. Moreover, due to the relatively early formation and relative stability of carbonylated proteins, this biomarker has some advantages over the measurement of other oxidation products. 3-Nitrotyrosine, the main product of tyrosine oxidation, may be produced either within a polypeptide or in free tyrosine residues. This modification can be generated through several pathways, including the reaction with ROS and RNS, such as ONOO- and NO_2 [35].

In addition, the powerful strategy offered by the mass-proteomic approach makes it possible to achieve a high sensitivity and specificity in determining the oxidative modifications in selected proteins. In the context of redox proteomics, the major players are cysteine residues (S-glutathionylation and GSH/GSSG disulphide), antioxidants (SOD, CAT and glutathione peroxidase), ROS-generating enzymes (nitrogen oxide, myeloperoxidase, xanthine oxidase and nitric oxide synthase), as well as the transcription factors involved in their regulation [36].

Reversible protein S-glutathionylation can occur under physiological conditions, within redox signalling pathways, or as a result of GSH antioxidant activity through the reduction of oxidized cysteine residues and the formation of mixed disulfide protein-glutathione. The measurement of S-glutathionylation of functionally important proteins is also a promising biomarker. A simpler approach is to analyse S-glutathionylation of proteins in circulating cells. In addition, GSH acts as

an antioxidant defence system due to its ability to scavenge ROS through its reversible oxidation to GSSG. The measurement of GSH, GSSG and their ratio in blood has been considered an index of the redox status in the whole organism [37]. However, GSH and its oxidized form of GSSG are not reliable biomarkers of oxidative stress because of the presence of some methodological artefacts. For instance, sample acidification for protein precipitation leads to an increase in GSSG levels [38].

Some ROS-generating enzymes can be found in blood circulation and, thus, can be used as markers of oxidative stress. The most important antioxidant enzymes are SOD, CAT and glutathione-dependent enzymes, such as GPX, glutathione reductase and glutathione transferase. SODs are a family of enzymes that catalyse dismutation of superoxide into oxygen and H_2O_2. SOD activity can be measured by analysing the inhibition in the rate of reduction of a tetrazolium salt by O_2^- generated through the xanthine/xanthine oxidase enzymatic system [39]. CAT, which catalyses the conversion of H_2O_2 into water and oxygen, is a homotetrameric protein containing four iron hemes and is largely located in the peroxisomes. However, to understand the contrasting results in human studies, methodological considerations must be made. In a meta-analysis, decreased activities of SOD and GPX were observed in the plasma/serum of postmenopausal women with osteoporosis, but the activities of SOD in erythrocytes and of CAT in plasma/serum were not statistically different from those in the control group. Therefore, we need to consider many types of indices rather than one aspect [24].

With improvements in the body antioxidant status, useful antioxidant indicators applied in human intervention studies to measure the levels of ingested antioxidants (by foods or supplements) must be reasonably stable, present in an easily accessible specimen and their measurement should be cost-effective [40]. It has been suggested that the bias of each method can be overcome by using more than one criterion. In this context, indices of the redox status have been proposed [41]. The OXY-SCORE is computed by subtracting the protection score (GSH, alpha- and gamma-tocopherol levels, together with antioxidant capacity) from the damage score (plasma free and total MDA, GSSG/GSH ratio and urine F2-isoprostaglandins) [42]. On the other hand, the "global oxidative stress index" (Oxidative-INDEX) is calculated by subtracting the OXY (the antioxidant capacity measured with the OXY adsorbent test) standardized variable from the ROM (the reactive oxygen metabolites measured with d-ROM) standardized variable. These scores are adjusted according to cardiovascular diseases, age, sex and smoking habits. The Oxidative-INDEX has been successfully used in human intervention studies with antioxidants [43].

The evaluation of antioxidant function is a comprehensive task and cannot scientifically and comprehensively evaluate the antioxidant capacity of a substance using only a single index. In this context, it has been suggested that the disadvantage of a single method could be reduced by using multiple indices of oxidative stress that include more than one marker.

CONCLUSIONS

This study has practical significance, which can provide a reference for the revision of the evaluation standard of antioxidant function of health-promoting food products. This allows for a more comprehensive evaluation of the antioxidant function of health-promoting food products and the discovery of more effective health-promoting food products.

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