Antioxidant potential of selected supplements *in vitro* and the problem of its extrapolation for *in vivo*

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Abstract

Introduction: antioxidants, free radicals and oxidative stress have been studied extensively for quite some time but their role in diseases and their prevention has not been clearly determined. Because commercial antioxidants do not need to pass clinical tests in order to be sold over the counter we have decided to test the antioxidant potential of different commercial preparations with the antioxidative properties.

Methods: pH, rH and oxidant-reduction potential of different preparations in aqueous solution was measured. Afterwards antioxidant potential using FormPlus® after adding the preparation to human blood as a more complex environment with different homeostasis mechanisms was determined.

Results: all the results showed expected change compared to the control but the results in aqueous solution did not match the results obtained from the human blood, as was expected.

Conclusion: from the experiments it can be concluded that while the preparations did show antioxidant activity, it is very difficult and even wrong to predict the antioxidant potential of an antioxidant preparation added to human blood, let alone in a living organism, based just on the results obtained in aqueous solution. Further possibilities for research include more extensive studies of antioxidant preparations in more complex environment and last but not least in test organisms or in human trials.

Keywords: antioxidants, oxidative stress, reactive oxygen species, food supplements

Introduction

Oxidative stress in a physiological setting can be defined as an excessive bioavailability of reactive oxygen species (ROS), which is the net result of an imbalance between production and destruction of ROS (with the latter being influenced by antioxidant defenses) (1). Oxidative stress is the direct consequence of an increased generation of free radicals and/or reduced physiological activity of antioxidant defenses against free radicals. The direct consequence of oxidative stress is damage to various intracellular constituents. In recent years oxidative stress has been implicated in a wide variety of degenerative processes, diseases and syndromes, including the following: mutagenesis, cell transformation and cancer; atherosclerosis, arteriosclerosis, heart attacks, strokes and ischaemia/reperfusion injury; chronic inflammatory diseases, such as rheumatoid arthritis, lupus erythematosus and psoriatic arthritis; acute inflammatory problems, such as wound healing; photo-oxidative stresses to the eye, such as cataract; central-nervous-system disorders, such as certain forms of familial amyotrophic lateral sclerosis, certain glutathione peroxidase-linked adolescent seizures, Parkinson's disease and Alzheimer's dementia; and a wide variety of age-related disorders, perhaps even including factors underlying the aging process itself (2). In order to understand oxidative stress, a brief introduction to the free radical formation and antioxidative defense will be presented. By definition, free radical is any chemical species which contains one or more unpaired electrons and can exist on its own (1). Eventhough that the reactions which free radicals are formed in seem simple *in-vitro*, the situation *in vivo* is much more complicated. In

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a living organism many chemical reactions happen at the same time, they intertwine and influence eachother, so grasping and researching the importance of free radicals in-vivo is quite difficult (3). It is not only obvious that free radicals are formed *in vivo* and that we cannot completely stop their formation but also that their chemichal features make them very reactive and unselective for the reactions they get involved in. Because radicals form in the presence of oxygen in aerobes we could generalize that when it comes to free radical chemistry oxygen is a harmful substance (4). Because of this fact humans and other aerobes have antioxidant mechanisms which help us minimise those effects. To protect against damage by ROS, all biological systems have evolved complex antioxidant systems composed of low molecular-weight compounds (such as glutathione and vitamin E) and enzymes, such as catalase, superoxide dismutase and glutathione peroxidase. Antioxidant is any substance which in concentrations smaller than that of a substrate slows down or prevents the oxidation of this substrate (1). Antioxidants can be endogenous or exogenous but in principle there is complex and cumulative effect when it comes to antioxidant protection and balance. Since endogenous antioxidant mechanisms cannot be deliberately increased researchers are focusing on the methods to increase the intake of exogenous antioxidants. Exogenous antioxidants, which are included in dietary supplements, also known as a food or nutritional supplements, are preparations intended to provide nutrients such as vitamins, minerals, fiber, fatty acids or amino acids that are either missing or not consumed in sufficient quantity in a person's diet. The idea that antioxidant supplements, such as Vitamin C, Vitamin E, lipoic acid and N-acetylcysteine, might extend human life stems from the free radical theory of aging (5, 6). Antioxidants are necessary for organisms living with a high $^3O_2$ concentration becaue they lessen the intensity and frequency of oxidative stress. Diet-derived antioxidants might be important agents in disease risk reduction, and might be beneficial for human health. When the balance between available antioxidants and the free radicals is ruined the organism comes in to a state of oxidative stress (1, 7). This might happen because of many reasons and might not even be noticed in the short term. Noticable problems in the organism arise when this state lasts for a while. Although the solution for oxidative stress seems obvious-just fix the ratio between the radicals formed and the antioxidants available-different studies have gained different results. Observational epidemiological studies provide the basis for relating the intake of vitamin E rich food to decreased incidence of risk of mortality due to cardiovascular diseases (8). However, the results from large-scale intervention studies on antioxidant supplements are inconclusive, reporting adverse, as well as beneficial, or no effects at all (9-20); e.g. daily supplementation with α-tocopherol (21, 22). Human intervention studies in which smoking male volunteers were exposed during 5-8 years to daily supplementation with vitamin E did not reveal any effect on the overall mortality of male smokers, but did show increased mortality resulting from hemorrhagic stroke (1, 23). On the other hand, impoverishment of the soil (resulting from the abnormal exploitation of the soil itself, acidic rains, increasing desertification, pollution, etc.), the often uncontrolled use of pesticides, the processes of refinement of vegetables, and the processes of transformation, storage and even the cooking of foods, can affect the antioxidant content of fruits and vegetables (24, 25). Besides, in most countries of the world the consumption of fruit and vegetables is below the minimal level of 400 g per day advised by WHO and FAO (26). The addition of different food supplements to the diet seems to be, besides consumption of fruit and vegetables for different reasons and especially in different clinical conditions, a need as well. Therefore, as a precaution, many nutritionists today suggest the indiscriminate use of antioxidants. However, the use of antioxidant supplements should be limited only to documented cases of oxidative stress and supplements should be safe and with proven health effects (27-29). The problem is that vitamin supplements do not have to pass all the tests that medicines do. While medicines need *in vitro* and *in vivo* studies, pre-clinical and many phases of clinical tests before they are approved for the use on people, vitamin supplements' activity and safety is not as vigorously tested so their effects and side-effects are easily questioned. Today consumers can find many products freely

accessible on the market claimed to posess antioxidative properties. The growing market of supplements and a less restrictive regulatory environment creates the potential for selling supplements with no in *vivo tests* done about their effectiveness and health effect. In the USA surveys show that more than half of the U.S. adult population uses food supplements. In 1996 alone, consumers spent more than $6.5 billion on dietary supplements. FDA or other similar institutions do not authorize or test dietary supplements since they are not intended to diagnose, cure, mitigate, treat, or prevent diseases. The manufacturer must just prove that new ingredient can reasonably be expected to be safe. In Slovenia "Pravilnik o prehranskih dopolnilih" defines food supplements as foods which are used to complement normal and diverse diet. The doses of substances in them must be expressed in percent of recommended daily allowance (%RDA). "Pravilnik o razvrstitvi vitaminskih in mineralnih izdelkov za peroralno uporabo, ki so v farmacevtskih oblikah, med zdravila - (Ur.l. RS, št. 83/2003; Ur.l. RS, št. 86/2008)." classifies certain food supplements as medicines. Those are substances which exceed the RDA or substances that are publicised as medicines (either for treatment or the prevention of the diseases). Besides these rules other conditions in terms of valid research are not included. Although the measurements and analyses of the food supplements in Slovenia have not been made there was a study conducted about the use of vitamin and mineral supplements in the Slovene population. The study of Poljšak et al., (30) showed that most of the people asked eat at least one meal of fruit or vegetables a day and that 72% of people think that adding the supplements is not necessary if one eats vegetables and fruit regularly. In contrast to this two thirds of people in the survey stated that they do use food supplements, half of them only in extreme cases (e.g. disease). Most commonly used supplements were A+C+E and multivitamins (30). More than half of them buy the products in the pharmacy. The most common reason for using the supplements is boosting the immune system. Number one source of information about the products is the media. A similar study, with similar outcome was performed also among Sarajevo inhabitants (31). Considering the formation of free radicals, the importance of antioxidant mechanisms, oxidative stress and the lack of testing of antioxidant supplements we have decided to research the effectiveness of the 4 synthetic and 2 natural products with antioxidant properties. First oxidation-reduction potential of all substances was measured to estimate the antioxidant »properties« of selected products *in vitro* in water solution by measuring pH and oxidation-reduction potential (ORP) of a substance. Then substances were injected in human blood, which is a far more complex environment. We expected that the added supplements will lower the concentration of free radicals in the blood and that the antioxidant potential will be higher compared to antioxidative potential measured as ORP in water solution and we proposed that all tested products will have a higher antioxidative potential in the samples compared to the control.

Methods

Preparation of solutions

The supplements we used were supplements containing vitamins A+C+E and selenium, only vitamin C, multivitamin supplement, Active H, coffee, green tea, water soluble Q10. All are available over the counter in pharmacies. The dillutions were prepared as follows:

Preparation of Active H, vitamin C, selen+ ACE, multivitamine supplement and Q10

We dissolved one tablette of either one of the supplements in 250 ml of distilled water. In this solution we measured pH and ORP. Afterwards we took away 5 ml and put it in a separate erlenmaier flask and added distilled water to 100 ml then we pipetted 10 µl from this mixture and added it to 0.2 ml of human blood.

Espresso coffee, green tea

250 ml of coffee or tea was made. We took away 5 ml and put it in a separate erlenmaier flask and added distilled water to 100 ml. Then we pipetted 10 µl from this mixture and added it to 0.2 ml of human blood. It is important to prepare each sample with blood right prior to measuring the potential, since different time of the interaction of supplement with blood might change the result due to the oxidation and coagulation of the blood.

We took 5 ml venous blood from a volunteer, and we put it in a test tube without any anticoagulants.

Measurement of ORP, pH and determination of rH

We measured the oxidation-reduction potential (ORP) and pH with the method precisely described in the article (32). These measurements were obtained in water solutions, not blood samples. Briefly, for the measurement of oxidation-reduction potential (ORP) and pH levels the simultaneous use of three instruments was performed: namely Inolab WTW pH meter, HACH Sension pH meter, HACH Sension ORP meter and Greisinger electronic ORP meter. All measurements were performed in 250 ml cup, previously mixed, at room temperature 25^{0}C. The final measured levels of pH and ORP were read in mV. The criterion for the reaction capability of a compound are oxidization/reduction potentials in mV. Reduction potential (also known as redox potential, oxidation/reduction potential or ORP) is the tendency of a chemical species to acquire electrons and thereby be reduced. Each species has its own intrinsic reduction potential; the more positive the potential, the greater the species' affinity for electrons and tendency to be reduced. pH of the solution is the criterion of concentration of free positive hydrogen ions in the solution. The use of rH gives a hydrogen proton-unbiased look at the absolute reducing potential of a compound, eliminating the effect of pH in the ORP measurement. It is a true indication of a compounds reduction potential capacity. The shifts in rH can be used to quantify the reducing ability and energy reserves of the compound. The rH level is the criterion for the state of reduction or oxidation in which is the compound, it is also the indicator of the probability that the compound will react with the free radical. The direct use of pH and reduction potential measurements (ORP) gives an indication of the probability of a compound to act as an antioxidant (33, 34). pH is the logarithm (base ten) of the molar concentration of hydrogen ions in a solution and it tells us whether the solution is acidic or basic. Redox potential depends also on the pH of the solution. ORP and pH can be used to calculate rH which uses both variables together to predict the likelyhood of the substance reacting with the free radical therefore acting as an antioxidant. Lower rH means that the substance is rich in hydrogen, while high values mean it contains more oxygen. rH of biological liquids should contain more hydrogen than oxygen quantitatively put rH should be below 28 (33, 34).

Nernst equation and rH

Because of the interaction of protons at the changes of pH oxidation-reduction potential may be biased by the pH and vice versa. For this reason the variation of Nernst equation (Equation [1]) was used, which is an effective way for measuring the reductive potential of a compound, which is given by the level of rH. This is the logarithmic value and is the criterion for absolute reductive potential.

$$E_h = 1.23 - \frac{RT}{F} pH - \frac{RT}{4F} \ln \frac{1}{P_0} \qquad [1]$$

E_h in the equation is the measured reductive potential (mV), F is the Faraday constant (the charge per a mole of electrons), equal to $9.6485309 \bullet 10^4$ C mol^{-1}, R is the universal gas constant, equal to 8.314510 $J \bullet K^{-1} \bullet mol^{-1}$ and T is the temperature in Kelvin. (Kelvin = 273.15 °C). The value 1.23 in the equation is the potential of oxygen at one atmosphere (101.235 kPa) 1.23 V higher than in the compound at the same pH. The level of rH is explicitly defined as the negative logarithm of oxygen pressure, P_o (equation [2]).

$$\mathrm{rH} = \log P_0 \qquad [2]$$

rH is the "absolute indicator of the reductive potential" of a substance (33-35). It shows then concentration of active hydrogen ions, rH can be determined indirectly with the determination of ORP and pH. The formula for its reckon was already discovered in 1923 by Clark (35) (remodelled Nernsto's equation), but only in later years it is gaining full value at studying processes in living beings. Basically it is a complicated logarithmic formula, but in practice (for measurements at 25 degrees Celsius) a simplified formula is used (equation [3]):

$$\mathrm{rH} = \frac{(ORP + 204)}{30} + 2 \cdot \mathrm{pH} \qquad [3]$$

Determination of total antioxidant capacity and the amount of free radiclas in human blood

The apparatus used for measuring the total antioxidant capacity and the amount of free radicals is called FORMplus® version 1.0. manufactured by Callegari. The two tests used for determination of oxidative stress in human blood were FORD (free oxygen radicals defence) and FORT (free oxygen radicals testing). The principle of FORD test is the use of free radicals which are formed from the reagents before adding of the blood sample and the change of the absorbance of light passing through the sample. This absorbance is proportional to the concentration of antioxidants in the added blood sample. In the presence of an acid buffer (pH=5.2) and an oxidant (FeCl3) the chromogen (amine derivative) forms a stable coloured compound (cation), which the machine detects at 505 nm wavelength. Antioxidant compounds (AOH) reduce the cation which causes discoloration of the solution. FORD test results of antioxidant concentration in the sample are given in the equivalent concentration of trolox, which is a water soluble vitamin E. FORT test principle is based on the fact that transition metals such as iron can catalyse the formation of free radicals in the presence of hydroperoxides. These free radicals are then trapped by an amine derivative which changes colour and is detectable at 505 nm. The intensity of the colour correlates directly to the amount of radicals in the solution. The results are given in the concentration of hydrogen peroxide. Because the apparatus measures the variables only in certain ranges, the supplements must be appropriately diluted in order to satisfy the ranges. Since the measuring FORD and FORT of the blood together with the antioxidant is not the standard procedure for using this apparatus, we had to design an experiment which would give results in the range of the machine. This means diluting the antioxidant preparations to certain concentrations. It should be noted that this depends on the amount of free radicals and antioxidant potential of the blood alone and that the dilution has to be adapted for each sample of blood.

Results

pH and ORP were measured in an aqueous solution, rH was calculated from pH and ORP using the formula described in methods. Table 1 shows the pH of the solutions of antioxidant supplements, coffee and green tea. pH is the logarithm (base ten) of the molar concentration of hydrogen ions in a solution and it tells us whether the solution is acidic or basic. It is evident that pure vitamin C has the lowest pH. Except for the active H solution which also has the highest pH from the samples, all other solutions are acidic. Reduction potential (also known as redox potential, oxidation/reduction potential or ORP) is the tendency of a chemical species to acquire electrons and thereby be reduced. Each species has its own intrinsic reduction potential; the more positive the potential, the greater the species' affinity for electrons and tendency to be reduced. The lowest ORP (oxidation-reduction potential) was measured in active H being very negative, the highest in pure vitamin C. Except fot Active H all other values are positive. The use of rH gives a hydrogen proton-unbiased look at the absolute reducing potential of a compound, eliminating the effect of pH in the ORP measurement. It is a true indication of a compounds reduction potential capacity. The shifts in rH can be used to quantify the reducing ability and energy reserves of the compound. The rH level is the criterion for the state of reduction or oxidation in which is the compound, it is also the indicator of the probability that the compound will react with the free radical. All values of rH are between 14 and 20. Again the lowest rH value was obtained in active H solution and selenium+ ACE, the highest in the multivitamin and vitamin C samples. Lower rH values mean that the supplement should have the highest antioxidant potential in vitro. From this result it

TABLE 1. Determination of pH, rH, ORP of selected solutions with antioxidant properties

	pH	ORP	rH
coffee	5.11	-15.5	16.98
Q10	3.70	39	16.50
Green tea	5.77	31.9	15.50
Vit c	4.22	-8.5	19.40
Ace + selenium	3.89	98.9	14.95
Multivitamin	3.72	6.2	17.89
Active H	7.66	-763. 5	14.45
Pure vitamin C (60mg)	2. 80	141	17.10

TABLE 2. FORD and FORT tests

	FORD** (mmol/Ltrolox)	FORT** (mmol/L H_2O_2)
Control sample	1.26	4.04
Vit c	1.53*	2.26
Multivitamin	1.44*	2.19
Q10	1.24*	2.87*
Selen + ACE	1.54*	2.53*
Green tea	1.62	3.52
Coffee	1.62	3.08
Active H	1.56	3.15

*result after diluting the original sample 10 times
** standard deviation of parallel samples was less than 5%

could be concluded that Active, A+C+E+selenium and green tea have ten times or even higher "antioxidant" potential than other compounds tested and this could offer greatest protection against free radical damage if used as supplements. All the samples in FORD test (measurement of total antioxidant potential) had higher result than the control sample, which means that the antioxidant potential of all tested substances was higher when adding it to the blood sample. The lowest potential was measured in active H. Vitamin C, multivitamin, Q10 and selenium+ACE preparations had to be additionally diluted so that the result could be obtained. This means that they had the highest antioxidant potential when added to the sample of blood, selenium+ ACE having the highest result among all samples. It should be noted that multivitamin and Q10 also contained 60 mg of vitamin C. This means that the highest results could be attributed to the presence of vitamin C, since all other antioxidants (e.g. vitamin E, beta carotene) are lipid soluble and therefore work when contained in a biologically active membrane. All the samples had lower result than control sample in FORT test, which means that adding the supplements to the blood sample, causes less H2O2 to remain or to be formed in the blood. This means that compounds tested did not form extra H2O2 which would indicate their prooxidative properties. Q10 and selenium + ACE had to be additionally diluted because original concentrations lowered the presence of H2O2 to the amounts undetectable by the spectrometer. After dilution the lowest concentrations of peroxide remained in the sample containing Q10. When comparing the results of pH, ORP and rH measured in aqueous solution of the supplements and the results obtained with FORD and FORT tests measured in blood, different values were observed. Blood is a more complex mixture and thus more important when extrapolating data for in vivo. We can see that predicting the most efficient antioxidant just by using values obtained in the aqueous solution is not only oversimplified but can also give different or even misleading results. While selenium + ACE had very promising results in rH values and also FORD and FORT tests, the Active H preparation promises the most with the rH value, but has poor functioning and results when added to the blood and measured with FORD and FORT tests.

Discussion

Antioxidant potential was higher compared to the control in all the preparations of blood containing added antioxidant, some of them had to be diluted to lower concentrations of antioxidant in order to get measurable results. These could be noted as the most effective antioxidants among the compounds tested. The most promising was selenium + ACE. The presence of free radicals in the blood was lowered by all antioxidants used and some solutions had to be additionally diluted. The preparation that scavenged the most free radicals was Q10, which also contains vitamin C. Comparing the results of pH, ORP and rH measured in aqueous solution of the supplements which predicted antioxidant potential in a water solution and the results obtained with FORD and FORT tests measured in blood we can see that predicting the most efficient antioxidant just by using values obtained in the aqueous solution is not only simplified but can also give different or even misleading results. The results predicted that Active H was the most powerful antioxidant with the lowest rH value. The Active H preparation promises the most with the rH value, but has poor functioning and results when added to the blood and measured with FORD and FORT tests on the other hand selenium + ACE supplement predicted good antioxidative properties when estimating rH value which were confirmed also with FORD and FORT tests. However Q10 supplement has a higher rH value but was quite efficient in FORD and FORT tests.

The main limitation of the study is that the absorption, metabolism, volume of distribution and excretion of the supplements were not considered since the antioxidants were added directly to the blood.. For this reason there is discrepancy between *in vitro* and *in vivo tests*. Besides it should be stressed that the results of epidemiological studies in which people were treated with synthetic antioxidants are inconclusive and contradictory, providing findings that prove either a beneficial effect, no effect, or a harmful effect of the synthetic antioxidant supplements. None of the major clinical studies using mortality or morbidity as an end point has found positive effects of supplementation with antioxidants such as vitamin C, vitamin E or β-carotene (9-13, 16). A simple experiment was performed to test wheter selected supplements on Slovenian market really posess antioxidative properties (determination of redox potential) and whether their antioxidative properties differ in water solution as well as in human blood (more complex matrix). The results from FORD and FORT tests show that there is a sinergistic effect between blood and added supplements, but as said, this sinergism is very complex and mechanisms unknown.

Conclusion

The testing of antioxidant potential of the patient's blood using FORD and FORT tests would be useful for everyday doctor's practice, since many diseases are the cause or consequence of oxidative stress and the tests are quite simple and quick to do. For example people with diabetes, rheumatoid arthritis, heart stroke and cancer could routinely be tested and different measures could be taken to lessen the oxidative stress besides of course controlling the basic disease as a priority. Future studies should be conducted *in vivo* but one should be aware of the important fact and the main difficulty which is that eventhough exogenous influences for the production of free radicals and intake of antioxiant supplements can be strictly controlled there are still many more endogenous proccesses which differ between individuals and are difficult, if not impossibe, to control for the purposes of designing the optimal *in vivo* experiment. Only well prepared and conducted clinical studies on human volounteers could reveal the true importance of food supplements with antioxidative properties on public health.

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Competing interests

The authors declare that they have no financial and personal relationships with other people or organizations that could inappropriately influence this work.

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