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Effects of flavonoid extract Enzogenol® with vitamin C on protein oxidation and DNA damage in older human subjects

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Abstract

In this study a 12 week clinical trial was conducted with Enzogenol®, a commercially available proanthocyanidin-rich flavonoid extract derived from the bark of *Pinus radiata* that was formulated with vitamin C. The study was to determine whether the oxidative injury markers of protein and DNA damage could be affected by the product. Twenty four (14 males and 10 females) subjects aged between 55-75 years completed the study. The group was given a twice daily dose of 240 mg of Enzogenol® and 120 mg vitamin C for 12 weeks and blood samples were collected at the start of the study before supplementation, 6 weeks and 12 weeks. Plasma samples were analysed for protein carbonyl concentrations as a measure of protein oxidation by an ELISA method. Isolated peripheral blood mononuclear cells were analysed for DNA damage using the alkaline comet assay. Protein carbonyl concentration reductions were highly significant after 6 and 12 weeks of supplementation. DNA damage reduction, as measured by the comet assay, was not significant after 6 weeks but highly significant after 12 weeks of supplementation.

Keywords: Flavonoid; Enzogenol®; Protein oxidation; DNA damage; Pine bark; Proanthocyanidin; Antioxidant

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1. Introduction

Free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated as by-products of normal cellular metabolism [1,2]. Their deleterious effects are minimized *in vivo* by the presence of antioxidant systems, both enzymatic and scavenging [2]. However, if these damaging species are ineffectively scavenged, they can interact with biological macromolecules, such as DNA, lipids and proteins, with potential threat to cellular function. According to the free radical theory of aging, loss of cellular function during aging is a consequence of accumulating sub-cellular damage inflicted by ROS [3].

Enzogenol® is a commercially available proanthocyanidin-rich, flavonoid extract derived from the bark of *Pinus radiata*. Characterisation and identification of the major constituents of *Pinus radiata* bark extract Enzogenol® has been extensive with over thirty components positively identified [4]. The most active are the ubiquitous proanthocyanidins, as monomers through to oligomers and some smaller chain oligomeric polyphenols and higher molecular weight polymers [4,5].

Bio-markers of oxidative damage to blood, such as oxidised lipids and proteins as well as damaged lymphocyte DNA, are thought to be useful indicators of oxidative stress that can be measured to indicate bio-availability and efficacy of antioxidant supplements. Protein carbonyl content is the most widely used marker of protein oxidation. Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury. They appear to be one of the first changes seen with low amounts of oxidant [6]. Furthermore the most prominent cell targets for the hydroxyl ($HO^{\cdot}$) free radicals are proteins [7]. The comet assay which measures DNA strand breaks at the level of single cells, is very easily applied to lymphocytes and therefore lends itself to human bio-monitoring studies [8]. It has become a standard technology for the measurement of oxidative DNA damage both *in vitro* and *in vivo* [9,10].

The antioxidant hypothesis postulates that supplementation with dietary antioxidants can alleviate the redox imbalance associated with disease. The potential use of dietary supplements for protection against the effects of oxidative stress and the progression of degenerative diseases and aging has been the subject of an increasing number of studies during the past two decades. Many studies have demonstrated the protective properties of the polyphenolic flavonoids. Antimutagenic, anticarcinogenic and immune stimulating properties of flavonoids have been reported [11,12]. The flavonoids are a large group of naturally occurring polyphenols found in fruits, vegetables, grains, bark, tea and wine that have proven *in vitro* free radical scavenging potential [9,13,14].

When Enzogenol® was tested in basic solution, the superoxide scavenging ability of Enzogenol® was 13 times more effective as an antioxidant than vitamin C. Furthermore, in aqueous and acidic solutions, Enzogenol® acted as a more potent antioxidant than vitamin C, catechin, and other bark, grape seed and grape skin extracts [5]. Phenolic compounds of *Pinus radiata* bark include catechin, epicatechin, quercetin, dihydroquercetin, taxifolin, phenolic acids, and procyanidin dimers, trimers, oligomers and polymers formed from catechin and epicatechin [15,16,17].

There is increasing evidence that tissue vitamin C in man is frequently present at sub-optimal concentrations [18]. Stabilisation of vitamin C by flavonoids is well established

[19,20] and there is evidence that dietary flavonoids increase the tissue concentrations of vitamin C in guinea-pigs [19]. Also another study shows that flavonoids significantly increases the absorption of vitamin C in man [18].

This paper reports the results of a pilot study on the dietary supplementation of Enzogenol® formulated with vitamin C in 24 healthy older people, that investigated the effect on protein carbonyl and peripheral lymphocyte markers of oxidative damage.

2. Materials and methods

2.1. Subjects

Twenty six subjects aged between 55-75 years with the mean age 64, were recruited for the 12 week study via an advertisement in the local newspaper. Selected subjects were non-smokers without any significant clinical disease entities and were not taking any other medications or food supplements. They were free from diabetes mellitus, treated hypertension, hormone replacement therapy, malignancy, and any serious concomitant disorder. A brief medical history was obtained from each participant and included age, ethnicity, past and current medical disorders and smoking history. Two individuals were withdrawn from the study. One for an unrelated medical condition, the other for non-compliance. Twenty four of the 26 subjects completed the study. The study was conducted by the Lipid & Diabetes Research Group, Christchurch Hospital and approved by the Ethics Committee, Christchurch Hospital, Christchurch, New Zealand [21].

2.2. Supplements

Capsules used in the study were supplied by ENZO Nutraceuticals Ltd. (Christchurch, New Zealand) and each capsule consisted of a mixture of *Pinus radiata* bark extract, Enzogenol® (120 mg) and vitamin C (60 mg). Subjects were instructed to consume 2 capsules prior to breakfast and 2 capsules prior to the evening meal with a glass of water, providing 480 mg of Enzogenol® plus 240 mg of vitamin C per day. The compliance of the subjects was checked by carrying out a count of capsules returned at 6 and 12 weeks.

2.3. Blood sampling

Fasting (12 hours) venous blood samples were collected into heparinised tubes for the carbonyl assay at baseline, and at 6 and 12 weeks of supplementation. Samples were immediately placed under refrigeration, and plasma was separated by centrifugation. Plasma samples were stored at −80°C until analysed. Each sample was analyzed for protein carbonyls in triplicate. Measurement of DNA oxidative damage was carried out on isolated peripheral lymphocytes at baseline, 6 weeks and 12 weeks. Isolation of peripheral lymphocytes was carried out based on the procedure published by Smith et al. [22]. Blood samples were collected into heparinised tubes. Peripheral lymphocytes were isolated from whole blood using density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Upp-

sala, Sweden) at 1900xg for 20 minutes. Cells were collected, washed twice with sterile PBS, and frozen in 8% DMSO at −80°C until analysed.

2.4. Protein carbonyl assay

Plasma samples were analyzed for protein carbonyl concentrations as a measure of protein oxidation by an enzyme-linked immunosorbent assay (ELISA) method of Buss [23]. This was carried out using a Protein Carbonyl Enzyme Immuno-Assay Kit (Protein Carbonyl Kit, Zentec, Dunedin, New Zealand), which performs the measurement of protein carbonyls in biological samples. Protein carbonyls were reacted with 2,4-dinitrophenyl hydrazine (DNPH) and then the protein was nonspecifically adsorbed onto an ELISA plate. The hydrazone adducts were detected with anti-DNP-biotin-antibody labelled with streptavidin-biotinylated horseradish peroxidase and reacted with chromatin. The absorbency was read at 450 nm directly after stopping the reaction by using plate reader (SPECTRAmax® 190, Molecular Devices, California). Each sample was analyzed in triplicate and samples were quantified by comparison with oxidized BSA standards [23].

2.5. Comet assay

The alkaline comet assay was performed as described by Singh et al. [24] with modifications previously described by Tice et al. [25]. Conventional frosted microscopic slides were dipped into hot 1.0% normal melting point agarose to one-half of the frosted area and the underside of the slide wiped to remove agarose. A 75 μl drop of 0.5% low melting point agarose (LMPA) at 37° C was mixed with ~10,000 cells in ~5-10 μL of Ficoll extract, and a cover slip was applied to spread the samples. After hardening, the cover slip was removed and a third agarose layer (75 μl LMPA) was added, the cover slip reapplied and removed after the agarose layer hardened. The slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, NaOH to pH 10.0, 1% Triton-100 and 10% DMSO) at 4° C for 1 hour to remove cell protein. They were then soaked in a coupling jar containing electrophoresis solution to unwind for 40 minutes and electrophoresed at a constant current of 300 mA, for 35 minutes. After electrophoresis, the slides were neutralised with Tris-HCl buffer at pH 7.5 by three washes for 5 minutes each followed with cold ethanol for 5 to 10 minutes and left to dry overnight. The slides were stained by placing 300 μl ethidium bromide solution (6 μg/mL) on each slide and covered with a coverslip for 20 minutes. They were then destained for 10 minutes in deionised water and viewed under an epifluorescence microscope (Zeiss epifluoresent) with an attached CCD camera and computer. Images were saved as electronic files and the Comets measured for comet tail length and tail moment based on the definition by Olive and Banath [26]. For each sample, 100 isolated comets were randomly selected and tail moments were measured.

2.6. Total antioxidant capacity

The total antioxidant capacity of Enzogenol®, grape seed, grape skin and green tea extracts were assessed by using the Oxygen Radical Absorbance Capacity (ORAC) assay.

The ORAC assay was carried out based on a method described by Cao et al. [27] using a Perkin Elmer LS50B Luminescence Spectrometer equipped with the four-position, motor driven, water thermostated, stirred cell holder. All the extracts tested were dissolved in distilled water and made up to 0.01 g/L solutions. The final results (ORAC value) are expressed using trolox equivalents antioxidant capacity (TEAC value) based on the area under phycoerythrin decay curve.

2.7. Statistical analysis

Statistical significances were determined by using paired t-test and t-test (SigmaStat software) for the data analysis. Differences in oxidative markers between gender were analysed by using the t-test and the remainder of the analysis was conducted using the paired t-test. Results are considered as significant when the calculated p value was less than 0.05 when compared between two means. In this study a paired t-test was used to compare three different tests, baseline values to values after 6 weeks of treatment and baseline values after 12 weeks of treatment and 6 weeks vs. 12 weeks. For the three points t-test, the Bonferroni correction was applied and the test is considered as significant if the output p value is less than 0.0167 (0.05/3).

3. Results

3.1. Protein carbonyls

The clinical trial was satisfactorily completed by twenty four (14 males & 10 females) healthy normal subjects aged between 55-75 with the mean age 64 years. The decreases in protein carbonyl concentrations were highly significant after 6 weeks ($p < 0.0001$) and 12 weeks ($p < 0.0001$) of supplementation (Fig. 1) compared to baseline. The formulated product reduced protein carbonyls, 51 and 42 percent after six and twelve weeks of supplementation respectively. However no significant difference was observed between 6 and 12 weeks of supplementation. Furthermore no statistically significant differences in protein carbonyl formation were observed between gender at 6 ($p = 0.541$) and 12 weeks ($p = 0.730$) of supplementation (Fig. 2).

3.2. DNA damage

The baseline, 6 week and 12 week samples were compared using paired t-test (Fig. 3). For each sample of 100 comets, the distribution of tail moments have a similar pattern for all 24 subjects, when sorted into rank order. This pattern can be expressed as chi-square distribution as shown by Bauer et al. [28], where a small fraction of the comets had very high tail moments. DNA damage reduction as measured by the Comet assay was not significant after 6 weeks ($p < 0.6900$) but highly significant after 12 weeks ($p < 0.0079$) of supplementation (Fig. 3). No statistically significant differences in DNA damage were observed, between gender, at 6 ($p = 0.721$) and 12 weeks ($p = 0.739$) of supplementation (Fig. 4).

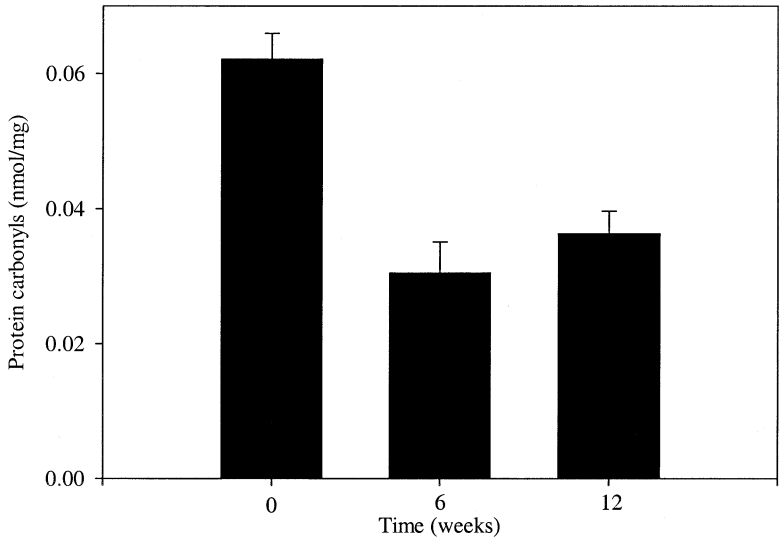

Fig. 1. The effects of Enzogenol® supplementation on plasma protein carbonyls in older subjects (n = 24). The average reduction of protein carbonyl concentration after 6 ($p < 0.0001$) and 12 weeks ($p < 0.0001$) are highly significant. The values are mean ± SEM

4. Total antioxidant activity

The ORAC results of Enzogenol® compared to other commercially available extracts are given in Table I, showing that Enzogenol® has a high ORAC value. Enzog-

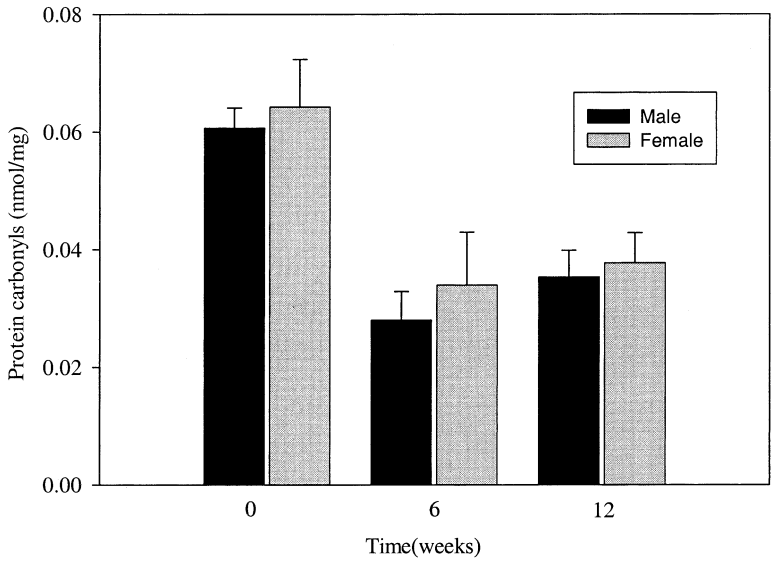

Fig. 2. The effects of Enzogenol® supplementation on plasma protein carbonyls in male (n = 14) and female (n = 10) older subjects. No significant differences between gender at 6 ($p = 0.541$) and 12 weeks ($p = 0.730$). The values are mean ± SEM.

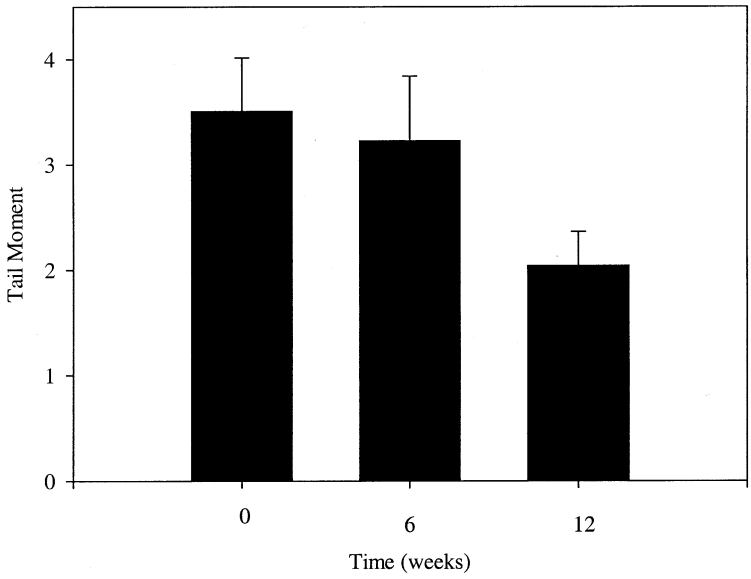

Fig. 3. The effects of Enzogenol® supplementation on DNA damage in older subjects (n = 24). The average reduction of DNA damage after 6 weeks ($p < 0.6900$) are not significant and after 12 weeks ($p < 0.0079$) highly significant. The values are mean ± SEM.

enol® is approximately 2 times more potent antioxidant than grape skin, grape seed and green tea extracts except purified grape seed extract which shows a similar antioxidant activity.

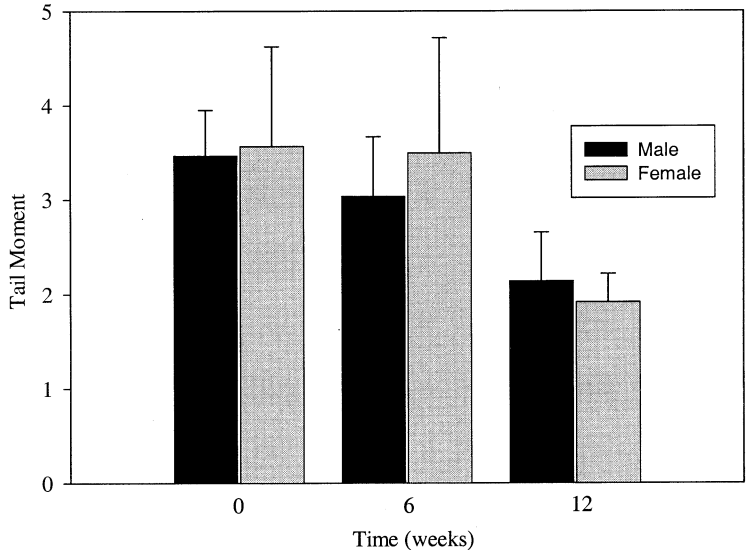

Fig. 4. The effects of Enzogenol® supplementation on DNA damage in male (n = 14) and female (n = 10) older subjects. No significant differences between gender at 6 ($p = 0.721$) and 12 weeks (0.739). The values are mean ± SEM.

Table 1
Total antioxidant activity of Enzogenol, grape seed, grape skin and green tea extracts[a] by Oxygen Radical Absorbance Capacity (ORAC) assay

Extracts	ORAC (μmole trolox/g)
Grape Seed Extract	2612 ± 92
Green Tea Extract	2871 ± 105
Grape Skin Extract	2260 ± 9
Grape Seed Extract (purified)	4765 ± 5
Enzogenol	4697 ± 18

[a] Values are expressed as means ± standard deviation of three determinations. All the extracts tested were dissolved in distilled water and made up to 0.01 g/L solutions.

5. Discussion

This study was carried out to find out the effect of the formulated product *Pinus radiata* bark extract, Enzogenol® in combination with vitamin C. The results indicate that both the oxidised protein marker and the DNA damage marker showed highly significant decreases over the course of the 12 week trial. However the time period of greatest change was in the baseline to 6 week period for the oxidised proteins and the 6 to 12 week period for the DNA damage decrease.

These results are consistent with the hypothesis that antioxidants can decrease markers of oxidative damage. This has been shown previously by the reduction of DNA damage in elderly people supplemented with fruit and vegetables extracts [22] and a correlation between DNA damage and low antioxidant levels in elderly subjects [29]. The proanthocyanidin-rich extract of *Pinus radiata* bark used in this study provides a highly concentrated source of antioxidants with strong activity that can be incorporated into supplements. The present study shows that Enzogenol®, a good source of phenolic phytochemicals, when used as a health supplement combined with vitamin C lowers DNA and protein oxidative damage.

The free radical scavenging ability of flavonoids can protect the human body from oxidative damage, which leads to the aging process and may cause many diseases including cancer, coronary heart disease [11,30]. Studies have shown that increasing levels of flavonoids in the diet could decrease cancer and heart disease [31,32]. Consumption of flavonoids is beneficial for people of all ages, however the elderly are at greater risk from protein and DNA damage than younger people. Ames and Shigenaga reported that, the level of metabolism is seven times higher in elderly rats than in adolescents, resulting in twice the number of DNA lesions [33].

McCall and Frei [34] recently summarized the scientific evidence for supplementation of humans with antioxidants on oxidative DNA, lipid and protein bio-markers and concluded that there was insufficient evidence that vitamin C supplementation can reduce oxidative damage in humans. However studies of cellular DNA have provided support for the protection of DNA from oxidation [35] although some studies indicated that vitamin C supplementation could increase DNA damage [36,37,38,39]. Increasing quercetin intake in the diet did not result in increased DNA protection *in vivo* [10] but the flavonoids quercetin and myricetin [9] and the isoflavonoids genistein and equol [40] did protect against DNA

damage *in vivo*. Supplementation of the diet with kiwifruit [41] or tomato [42] was noted to increase the ability of lymphocytes to resist oxidative damage against DNA but there has been no equivalent study on the effects of higher molecular weight proanthocyanidins.

There have been a very limited number of studies using protein carbonyls as a marker in nutritional supplementation trials. In a recent clinical trial [43], following 5 weeks supplementation with vitamin C (400 mg/day), plasma ascorbate levels increased but no significant effect on immunoglobulin carbonyl levels was observed. However at 10 and 15 weeks supplementation, carbonyl levels of immunoglobulin were significantly reduced in subjects with low baseline ascorbate but not in those with normal base line ascorbate. A study of selenium supplementation to pre-term infants, showed plasma protein carbonyl concentration did not differ significantly between the supplemented and unsupplemented groups [44]. In our study a significant reduction in protein carbonyl was observed during the first 6 weeks of the supplementation. This leads to the conclusion that the antioxidants give significant protection of body protein within a shorter period compared with DNA. A slight increase was observed in protein carbonyl from 6 to 12 weeks but statistically this is insignificant. It might be argued that this variation could be due to other factors such as recreational activities or diet during the trial.

The formation of carbonyl groups on amino acid residues as a result of free radical-initiated reactions is well documented [45]. The formation of carbonyl groups occurs during normal aging [46,47], diseases associated with aging and premature aging [48]. Even in native plasma, there is a trend towards higher carbonyl levels in smokers than in controls [49]. Carbonyl formation is increased by oxidative stress [50] and is associated with several diseases of humans including Alzheimer's disease, rheumatoid arthritis, and inflammatory bowel disease.

In this study we also evaluated the *in vitro* antioxidant activity of Enzogenol® and tested its scavenging activity against peroxyl radicals using the ORAC assay. Enzogenol® was found to be an efficient scavenger of peroxyl radicals. Results indicate that the ORAC value of Enzogenol® is closer to purified grape seed extract, which is also rich in proanthocyanidins, and much higher than grape skin and green tea extracts. The health benefit of various grape seed extracts and tea extracts are well studied [51,52]. This *in vitro* test indicates that Enzogenol® is a rich source of antioxidants.

Various authors have reported that the activity of the antioxidant enzymes such as superoxide dismutase [53] and glutathione peroxidase [54] do not change with aging. Further, no correlation was observed between catalase and glutathione peroxidase activities and the maximum life span of various species in some aging models. These results indicate that aging is not associated with a shortage of antioxidant enzyme protection in these models. It may be possible that other antioxidants that we consume play an important role in anti-aging. Particular emphasis has focused on polyphenolics (flavonoids) and phenolic acid compounds. Flavonoids have been shown to possess a number of biological effects, countering inflammatory, bacterial, viral, fungal, hormonal, carcinogenic, neoplastic and allergic disorders in both *in vitro* and *in vivo* systems [13]. Therefore optimal intake of antioxidants may aid in the prevention of some age related diseases.

The balance between free radical production/exposure and the antioxidant capacity of the body can be adversely affected by multiple factors. Air pollution, exposure to chemicals,

smoking, poor dietary habits such as low intake of fruits and vegetables and diets rich in fatty foods, smoking and alcohol consumption are potential factors contributing to the development of oxidative stress. The present open label study found that a proanthocyanidin rich, pine bark extract, Enzogenol® with vitamin C significantly reduced both protein and DNA oxidation, thus reducing oxidative stress. A double blind placebo controlled study on the protective effects of this pine bark extract against oxidative stress is warranted.

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References

[1] Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of NO with $O_2^{\cdot -}$. Am J Physiol 1995;268:L699.
[2] Griffiths HR, Lunec J. Molecular aspects of free radical damage in inflammatory autoimmune pathology. In: Aruoma OI, Halliwell B., editors. Molecular Biology of Free Radicals in Human Diseases, 1998, OICA International, pp. 327–366..
[3] Harman D. The aging process. Proc Natl Acad Sci USA 1981;78:7124–8.
[4] Gilmour I. Fighting free radicals. In: Duncan K, editor. The Enzogenol Story. Auckland New Zealand: The Pacific Scientific Press, 1998. p. 55–60.
[5] Wood JE, Senthilmohan ST, Peskin AV. Antioxidant activity of procyanidin-containing plant extracts at different pHs. Food Chemistry 2002;77:155–61.
[6] Ciolino HP, Levine RL. Modification of proteins in endothelial cell death during oxidative stress. Free Rad Biol Med 1997;22:1277–82.
[7] Lodish H, Baltimore D, Berk A, Zipursky SL, Matsudaira P, Darnell J. Molecular cell biology, 3rd ed. New York: W H Freeman and Co, 1995.
[8] Collins A, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, Raslova K, Vaughan N. Comet assay in human biomonitoring studies: Reliability, validation, and applications. Environ Mol Mutag 1997;30:139–46.
[9] Duthie SJ, Collins AR, Duthie CG, Dobson VL. Quercetin and myricetin protect against hydrogen peroxide induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. Mutat Res 1997a; 393:223–31.
[10] Beatty ER, O'Reilly DO, England TG, McAnlis GT, Young IS, Halliwell B, Geissler CA, Sanders TAB, Wiseman H. Effect of dietary quercetin on oxidative DNA damage in healthy human subjects. Br J Nutr 2000;84:919–25.
[11] Middleton E, Kandaswami C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: Harborne JB, editor. The flavonoids: advances in research since 1986. London: Chapman and Hall, 1993. p. 619–52.
[12] Middleton E. Biological properties of plant flavonoids: an overview. International J Pharmacog 1996;34: 344–48.
[13] Duthie SJ, Johnson W, Dobson VL. The effect of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimidines) and growth in human cells. Mutat Res 1997b;390:141–51.

[14] Johnson MK, Loo G. Effects of epigallcatechin gallate and quercetin on oxidative damage to cellular DNA. Mutat Res 2000;459:211–18.
[15] Markham KR, Porter LJ. Extractives of Pinus radiata bark. N Z J Sci 1973;16:751–61.
[16] Porter LJ. Extractives of Pinus radiata bark. N Z J Sci 1974;17:213–18.
[17] Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, Pycnogenol. Free Rad Biol Med 1999;27:704–24.
[18] Jones E, Hughes RE. The influence of bioflavonoids on the absorbtion of vitamin C. IRCS J Med Sci 1984;12:320.
[19] Hughes RE, Wilson HK. Flavonoids: some physiological and nutritional considerations. Prog Med Chem 1977;14:285–301.
[20] Jones, E. Nutritional Significance of Flavonoids. Ph.D. Thesis 1983;University of Wales, UK.
[21] Shand, CB, Strey, C, Scott, R, Morrison, Z, Gieseg, S. Pilot study on the clinical effects of dietary supplementation with Enzogenol® a flavonoid extract of pine bark and vitamin C. Phytother Res. In press.
[22] Smith MJ, Inserra PF, Watson RR, Wise JA, O'Neill KL. Supplementation with fruit and vegetable extracts may decrease DNA damage in the peripheral lymphocytes of an elderly population. Nutr Res 1999;19: 1507–18.
[23] Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. Free Rad Biol Med 1997;233:361–66.
[24] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.
[25] Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutag 2000;35:206–21.
[26] Olive PL, Banath JP. Induction and rejoining of radiation-induced DNA single-strand breaks: "tail moment" as a function of position in the cell cycle. Mutat Res 1993;294(3):275–83.
[27] Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Rad Biol Med 1993;14:303–11.
[28] Bauer E, Pecknagel RD, Fiedler U, Wollweber L, Bock C, Greulich KO. The distribution of the tail moments in single cell gel electrophoresis (comet assay) obeys a chi-square (χ^2) not a gaussian distribution. Mutat Res 1998;398:101–10.
[29] Mendoza-Nunez VM, Sanchez-Rodriguez MA, Retana-Ugalde R, Vargas-Guadarrama LA, Altamirano-Lozano MA. Total antioxidant levels, gender, and age as risk factors for DNA damage in lymphocytes of the elderly. Mech Ageing Dev 2001;122:835–47.
[30] Winterbourn CC. Nutritional antioxidants: their role in disease prevention. N Z Med J 1995;108:447–48.
[31] Block G, Patterson B, Subar A. Fruit, vegetable and cancer prevention: a review of the epidemiological evidence. Nutr Cancer 1992;18:1–29.
[32] Hertog MLG, Feskens EJM, Hollman PHC, Katan MB, Kromhout D. Dietary antioxidants flavonoids and the risk of coronary heart disease: the zutphen elderly study. Lancet 1993;342:1007–11.
[33] Ames BN, Shigenaga M.K. Oxidants are a major contributor to aging. Ann NY Acad Sci 1992;663:85–96.
[34] McCall MR, Frei B. Can antioxidant vitamins materially reduce oxidative damage in humans? Free Rad Biol Med 1999;26:1034–53.
[35] Carr A, Frei B. Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J 1999;13: 1007–24.
[36] Anderson D, Phillips BJ, Yu T, Edwards AJ, Ayesh R, Butterworth KR. The effects of vitamin C supplementation on biomarkers of oxygen radical generated damage in human volunteers with "low" or "high" cholesterol levels. Environ Mol Mutag 1997;30:161–74.
[37] Singh NP. Sodium ascorbate induces DNA single-strand breaks in human cells in vitro. Mutat Res 1997;375:195–203.
[38] Anderson D, Phillips BJ. Comparative *in vitro* and *in vivo* effects of antioxidants. Food Chem Toxicol 1999;37:1015–25.

[39] Noroozi M, Angerson WJ, Lean MEJ. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. Am J Clin Nutr 1998;67:1210–18.
[40] Sierens J, Hartley JA, Campbell MJ, Leathem AJC, Woodside JV. Effect of phytoestrogen and antioxidant supplementation on oxidative DNA damage assessed using the comet assay. Mutat Res 2000;485:169–76.
[41] Collins BH, Horska A, Hotten PM, Riddoch C, Collins AR. Kiwifruit protects against oxidative damage in human cells and *In vitro*. Nutr Cancer 2001;39:148–53.
[42] Riso P, Pinder A, Santangelo A, Porrini M. Does tomato consumption effectively increase the resistance of lymphocyte DNA to oxidative damage? Am J Clin Nutr 1999;96:712–18.
[43] Carty JL, Beavan R, Waller H, Mistry N, Cooke M, Lunec J, Griffiths HR. The effects of vitamin C supplementation on protein oxidation in healthy volunteers. Biochem Biophys Res Comm 2000;273:729–35.
[44] Winterbourn CC, Chan T, Buss H, Inder TE, Mogridge N, Darlow BA. Protein carbonyls and lipid peroxidation products as oxidation markers in pre-term infant plasma: association with chronic lung disease and retinopathy and effects of selenium supplementation. Pediatr Res 2000;48(1):84–90.
[45] Stadtman ER. Metal ion catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. Free Rad Biol Med 1990;9:315–25.
[46] Oliver C, Ahn B, Moerman E, Goldstein S, Stadtman E. Age-related changes in oxidized proteins. J Biol Chem 1987;262:5488–91.
[47] Sohal RS, Ku HH, Agarwal S, Forster MJ, Lal H. Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. Mech Ageing Devel 1994;74:121–33.
[48] Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. Biocheml J 1997;324:1–18.
[49] Marangon K, Devaraj S, Jialal I. Measurement of protein carbonyls in plasma of smokers and in oxidized LDL by an ELISA. Clin Chem 1999;45(4):577–78.
[50] Reznick A, Packer L Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 1994;233:357–63.
[51] Yamakoshi J, Kataoka S, Koga T, Ariga T. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. Atherosclerosis 1999;142:139–49.
[52] Prior R, Cao G. Antioxidant capacity and polyphenolic components of teas: Implications for altering in vivo antioxidant status. Proc Soc Exp Biol Med 1999;220:255–61.
[53] Niwa Y, Ishimoto K, Kanoh T. Induction of superoxide dismutase in leukocytes by paraquat: correlation with age and possible predictor of longevity. Blood 1990;76:835–41.
[54] Santa Maria C, Machado A. Effect of development and ageing on pulmonary NADPH-cyt C reductase, glutathione peroxidase, glutathione reductase and thioredoxin reductase activities in male and female rats. Mech Ageing Dev 1987;37:183–95.