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The Effect of Tannic Acid on DNA Damage in Human Lymphocytes

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Introduction

It is well known that free radical damage is closely connected with the development of a wide range of degenerative diseases such as atherosclerosis, cataract, age-related macular degeneration, multiple sclerosis, and cancer[1,2]. Phenolic substances in natural products play an important role in protecting the organism against harmful effects of reactive oxygen species. The use of phenolic phytochemicals and herbal products for antioxidant purposes has been increased and a vast number of products have entered the markets all over the world. However some phenolic compounds are also reported to induce oxidative damage through the generation of reactive oxygen species[3]. Tannic acid (TA), present in many plant beverages and foods, such as herbal teas, beer, walnut, hazelnut and berries might acts as an antioxidant or a prooxidant depending on the concentrations of TA and the number and position of hydroxyl groups on TA. TA, found in food between 10-1300 ppm, is recognized as safe (Generally Recognized as Safe, GRAS). Tannins and TA have been listed as tentative carcinogens of Category I by the Occupational Safety and Health Administration (OSHA)[4].

Oxidative DNA damage may involve the breakage in single- and double-strands, base modifications, fragmentation of deoxyribose, formation of DNA-protein cross-links as well as abasic sites[5,6]. DNA strands breaks in eukaryotic cells can be detected by single cell gel electrophoresis (comet assay) which is commonly used for the assessment of protective effects of antioxidants on DNA damage in intervention studies with and without the addition of the repair enzymes endonuclease-III (Endo III), formamidopyrimidine N-glycosylase (Fpg), to characterize DNA lesions. The Fpg protein

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has been used widely for the detection of oxidative DNA base damage[7]. Fpg initiates the repair of oxidized bases by excising them and cutting the sugar-phosphate backbone of the DNA molecule. Thus additional strand breaks are induced at the location of oxidized base, causing DNA relaxation and migration. The detection of Fpg-sensitive DNA lesions revealed the presence of oxidized purine bases[8-11].

In the present study, the antioxidant capacity of TA was determined by the trolox equivalent antioxidant capacity assay and the effect of TA on DNA damage induced by H_2O_2 in human lymphocytes were investigated by the standard comet assay and the formamidopyrimidine-DNA-glycoslase (Fpg) modified comet assay.

Materials and Methods

Chemicals and reagents

The compound tannic acid used in the experiments was from Merck Chemicals (Darmstadt, Germany); the purity of the compound was 96 %. The other chemicals used in the comet assay and TEAC assay were purchased from the following suppliers. Normal melting agarose (NMA) and low melting agarose (LMA) were from Boehringer Mannheim (Germany); sodium chloride (NaCL), potassium chloride (KCL), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hydrogen peroxide (H_2O_2), sodium hydroxide (NaOH), and hydrochloric acid (HCL) were from Merck Chemicals (Darmstadt, Germany); heparine, dimethyl-sulfoxide (DMSO), ethidium bromide (EtBr), Triton X-100, phosphate buffered saline (PBS) tablets, bovine serum albumin, formamidopyrimidine-DNA glycosylase (Fpg), potassium peroxodisulphate, and 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid (ABTS) were from Sigma (St. Louis, MO); ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA-Na_2), N-lauroyl sarcosinate, and Tris were from ICN Biochemicals (Aurora, OH, USA), HPLC grade ethanol was from Fluka Chemie AG., Histopaque-1077 was from Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) and 6-hydoxy-2,5,7,8-tetramethylcroman-2-carboxylic acid (trolox) was from Calbiochem/ Roche.

Trolox equivalent antioxidant capacity assay (TEAC)

The TEAC method is one of the most often used methods for the determination of total antioxidant capacity[12-13]. It is based on the neutralization of the radical cation formed by a single-electron oxidation of a synthetic ABTS chromofore to a strongly absorbing $ABTS^{\bullet+}$ radical. The radical reacts quickly

with electron/hydrogen donors to form colorless ABTS. A decrease of the $ABTS^{\bullet+}$ concentration is linearly dependent on the antioxidant concentration including trolox as a calibrating standard. A mixture of 7 mM ABTS and 2.45 mM potassium persulfate (1:1, v/v) was incubated for 12 h at room temperature in the dark to form $ABTS^{\bullet+}$ radical. The working solution was diluted with ethanol to give an absorbance of around 0.8 at 734 nm. After preparing trolox standards and TA samples, they were mixed with equal volumes of ABTS solution and the decrease in absorbance was measured at 734 nm after 2 min. The trolox concentration is proportional to the change in absorbance of the ABTS solution. A standard calibration curve was constructed for trolox at 0.05 - 150 μM concentrations. 500 μl of TA solutions at concentrations of 0.05-150 μM in ethanol were mixed with 500 μl of ABTS. Samples were assayed in three replicates. The absorbance of the resulting oxidized solution was compared to that of the calibrated trolox standard.

Single cell gel electrophoresis (comet assay) and Fpg-modified comet assay

For each experiment, 5 mL heparinized (50 units/mol sodium heparin) whole blood was collected by venepuncture from a healthy 28-year-old non-smoker female donor not exposed to radiation or drugs. The replicate experiments were carried out with blood samples from the same donor collected at different time intervals.

Lymphocytes were isolated by Ficoll-Hypaque density gradient and washed with PBS[14]. Cell concentrations were adjusted to approximately 2 x 10^5/mL in the buffer. The cells were suspended in a total volume of 1 mL and each reaction contained 50 μL suspension (≈10^4 cells), varying micro liter amounts of TA dissolved in PBS at the concentrations of 0.05-100 μM. The cells were incubated for 30 min at 37 °C in an incubator together with untreated control samples. Control incubations contained PBS. After incubation the lymphocytes were harvested by centrifugation at 800 x g for 3 min at 4 °C. Oxidative damage was introduced by replacing the medium with PBS containing 0.05 mM concentration of H_2O_2, and the treatment was applied for 5 min on ice. Then the cells were harvested by centrifugation at 800 x g for 3 min at 4 °C. Again the cells were harvested by centrifugation at 800 x g for 3 min at 4 °C after washing with PBS. The lymphocytes were suspended in 75 μL of low melting point agarose (LMA) for embedding on slides. Cells were checked for viability by trypan blue exclusion test. This method is based on the principle that live cells possess intact *cell* membranes *excluding* certain dyes, such as *trypan blue. Cell viability* is calculated as the number of viable cells divided by the total number of cells within the grids on the hemacytometer.

The basic alkaline technique of Singh *et al*[15], as further described by Collins *et al*[10], was followed. Each microscopic slide had been each covered with 1% NMA at about 45 °C in Ca^{2+}-and Mg^{2+}-free PBS before the experiment. This layer was used to promote the attachment of the second layer. For the second layer, around 10000 treated or control cells mixed with 75 µL of 0.5% LMA were rapidly pipetted onto this slide, spread using a cover slip and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the cover slip, the slides were immersed in cold lysing solution (2.5 M NaCL, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10) with 1% Triton-X 100 and 10% DMSO added just before use for a minimum of 60 min at 4 °C but the cells treated with H_2O_2 and embedded on slides were immersed in other cold lysing solution.

The alkaline comet assay using formamidopyrimidine-DNA glycosylase (Fpg), lesion-specific enzyme was used to detect oxidized purines as a result of oxidative stress-induced DNA damage as described with some modifications[8]. The cell-agarose suspension slides were prepared as described above for the standard comet assay. After lysing, for the examination of levels of oxidized purine bases in lymphocytes, the slides were washed with enzyme buffer (40 mM HEPES, 100 mM KCL, 0.5 mM EDTA and 0.2 mg/mL bovine serum albumin) at room temperature and then incubated with Fpg protein (1 mg/mL in enzyme buffer for 30 min at 37 °C in an incubator and washed with the neutralizing solution. Then the slides were applied to electrophoresis.

The slides were removed from the lysing solution, drained and placed side by side avoiding space with the agarose ends facing each other nearest the anode in a horizontal gel electrophoresis tank. The tank was filled with fresh electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH pH 13) to a level approximately 0.25 cm above the slides. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali labile damage. Electrophoresis was conducted at a low temperature (4 °C) for 20 min using 24 V and adjusting the current to 300 mA by raising or lowering the buffer level and using a compact power supply (Power Pack P 25 Biometra Analytic GmbH). All of these steps were performed under dimmed light (tank was covered with a black cloth) to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were taken out of the tank. Tris buffer (0.4 M Tris, pH 7.5) was added dropwise gently to neutralize the excess alkali and the slides were kept for 15 min, and then the slides were kept for 5 min in 50%, 75%, and 95% alcohol, successively.

The dried microscope slides were stained with 20 µg/ml EtBr in distilled water (60 µl/slide), The slides were covered with a cover slip, placed in a humidified airtight container to prevent drying of the gel, and analyzed within 3-4 h.

The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize DNA damage, the slides were examined at 100x. DNA damage was expressed as tail intensity, tail migration, and tail moment. 100 cells from each of two replicate slides were assayed. Analysis was performed by one slide reader, thus minimizing variability due to subjective scoring.

Statistical analysis

Statistical analysis was performed by the computer program SPSS for Windows 15.0. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences was performed by least significant difference (LSD) test. The Kruskal-Wallis *H* test was used in comparing parameters displaying abnormal distribution between groups. The results were given as the mean ± standard deviation and *the P* values of less than 0.05 were considered as statistically significant.

Results and Discussion

Cell viability, as tested using trypan blue dye exclusion of each treated group, was more than 90 %.

TA demonstrated significant antioxidant capacity within the concentrations of 0.1-150 µM in a dose dependent with respect to ABTS diluted with ethanol giving an absorbance of around 0.8 at 734 nm. Within the concentrations of 1-10 µM, the antioxidant activity of TA was found to be significantly higher than the antioxidant activity of trolox (Figure 1). The trolox equivalent value of TA using the standard calibration curve of trolox was given in Table I. The trolox equivalent antioxidant capacity of TA increased in a dose dependent.

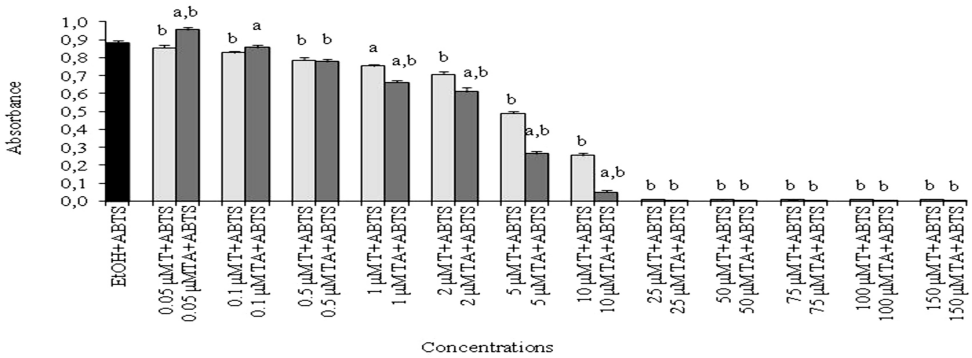

Figure 1

The antioxidant activity of tannic acid and trolox on ABTS. Results were given as the mean ± standard deviation. ap <0.05, tannic acid compared to trolox (0.05, 0.1, 0.5, 1, 2, 5, 10, 25, 50, 75, 100, and 150 µM); bp <0.05, tannic acid or trolox compared to EtOH+ABTS. TA, tannic acid; T, trolox.

TABLE I

The trolox equivalent antioxidant capacity of TA*

	TE (µM)
0.05 µM TA	1.65
0.1 µM TA	2.63
0.5 µM TA	3.42
1 µM TA	4.56
2 µM TA	5.07
5 µM TA	8.51
10 µM TA	10.63
25 µM TA	11.07

*Trolox equivalent value (TE) was calculated according to the trolox standart curve. TA, tannic acid.

The effects of TA on DNA strand breakage expressed as tail moment, tail migration, and tail intensity in human lymphocytes with or without H_2O_2 are shown in Figure 2. According to the data from three separate experiments, no additional DNA strand breakage in human lymphocytes was observed at the concentrations of 0.1-100 µM of TA. The numbers of damaged cells were significantly reduced when lymphocytes were incubated with 0.05 mM H_2O_2 and TA within the concentration range of 0.1-100 µM (Figure 2A, 2B, and 2C). However at all the concentrations studied, TA did not have a protective effect on oxidative DNA damage, the most oxidative DNA damage reducing effect was seen at 100 µM of TA.

As shown in Figure 3, DNA damage expressed as tail moment, tail migration, and tail intensity in the Fpg and H_2O_2-treated lymphocytes were found to be higher than the H_2O_2-treated lymphocytes. This result has indicated that DNA damage is the result of the increases in the levels of Fpg sensitive sites showing the oxidized purine base damage in the lymphocytes. It was found that within the concentration of 0.1-100 μM TA alone did not induce Fpg sensitive sites. However TA treatment reduced the Fpg sensitive sites induced by H_2O_2 in a dose dependent, moreover within the concentration of 5-100 μM TA, there were no differences of Fpg sensitive sites induced by H_2O_2 expressed as DNA tail moment and DNA tail migration

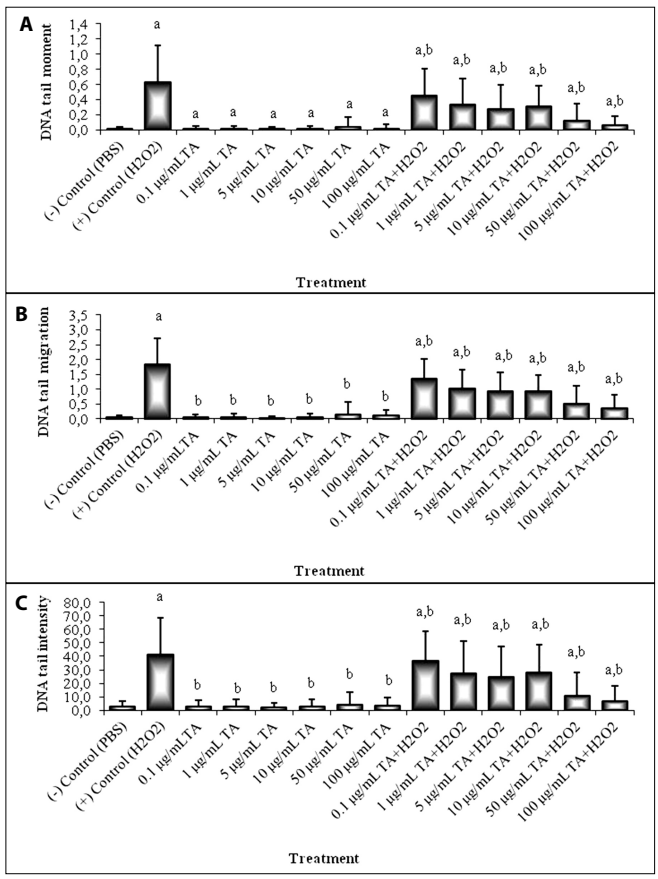

Figure 2

The effects of tannic acid on H_2O_2-induced oxidative DNA damage in human lymphocytes. DNA damages were expressed as DNA tail moment (**A**), DNA tail migration (**B**), and DNA tail intensity (**C**) in the lymphocytes of three separate studies. Results were given as the mean ± standard deviation. [a]p <0.05, compared with the negative control (PBS); [b]p<0.05 compared with the positive control (0.05 mM H_2O_2). TA, tannic acid.

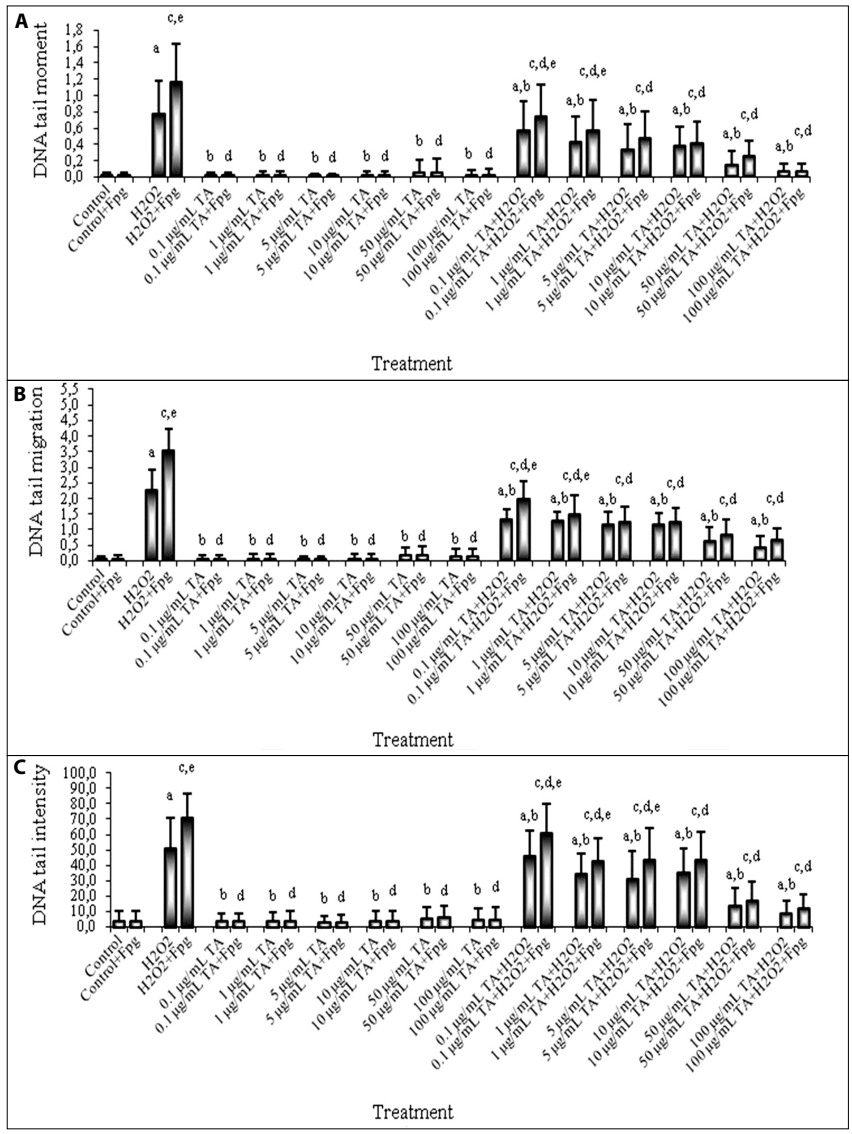

Figure 3

The effects of Fpg and tannic acid on H_2O_2-induced oxidative DNA damage in human lymphocytes. DNA damages were expressed as DNA tail moment (**A**), DNA tail migration (**B**), and DNA tail intensity (**C**) in the lymphocytes of three separate studies. Results were given as the mean ± standard deviation. [a]p <0.05, compared to negative control for the standard comet assay; [b]p <0.05, compared to positive control for the standard comet assay; [c]p <0.05, compared to negative control for the Fpg-modified comet assay; [d]p <0.05, compared to positive control for the Fpg-modified comet assay; [e]p <0.05, the standard comet assay was compared to the Fpg-modified cometassay.TA, tannic acid.

Phenolic substances play an important role in protecting the organism against harmful effects of reactive oxygen species. In recent years, many efforts have been focused on the safety and toxicity of phenolic substances found as natural dietary components. There is no sufficient information about the potential risk of these substances on human health. There are some conflicting studies that in *in-vitro* test systems, some herbal compounds are mutagenic and induce DNA damage[16].

Tannins (commonly referred to as tannic acid), water-soluble polyphenols, are present in many plant beverages and foods, such as herbal teas, beer, walnut, hazelnut, and berries. Tannins have been reported to be responsible for decreases in feed intake, growth rate, feed efficiency, net metabolizable energy, and protein digestibility in experimental animals. They have some physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease serum lipid level, cause liver necrosis, and modulate immunoresponses[16].

It has been suggested that tannins might be carcinogenic, as the incidences of certain cancers, such as esophageal cancer, which is related to consumption of tannins-rich foods. However, other reports indicated that the carcinogenic activity of tannins might be related to components associated with tannins rather than tannins themselves. Interestingly, many reports indicated negative association between tea consumption and incidences of cancers. Tea polyphenols and many tannin components were suggested to be anticarcinogenic. Many tannin molecules have also been shown to reduce the mutagenic activity of a number of mutagens[16]. Huang and Ferrao[17] reviewed antimutagenic activity of different component of tannins on different types of tumors, including esophageal, forestomach, duodenum, small intestine, colon, lung, liver, mammary gland, pancreas and skin of different animals.

Tannins seemed to exert anticarcinogenic activities at a certain concentration range and tumor-promoting activities while at concentrations beyond that range. The anticarcinogenic and antimutagenic potentials of tannins may be related to their antioxidative property in protecting cellular components from oxidative damages including lipid peroxidation, DNA single-strand breakage and formation of 8-hydroxydeoxyguanosine. Unfortunately, experimental results covering a wide range of concentrations are rare and incomplete. An animal model should be developed for further research in order to resolve such a contradiction[16,18-19].

Phytochemicals scavenge free radicals but also induce pro-oxidative reactions in the cell. But due to their diverse chemical structures, they are

likely to possess different antioxidant capacities[20,21]. During the reactions of tea polyphenols with free radicals, several oxidation products are formed[22-26]. TA was reported to act as an antioxidant or a prooxidant, depending on the concentrations of TA and the number and position of hydroxyl groups on TA. The degradation of 2-deoxyribose induced by 6 μM Fe (II) plus 100 μM H_2O_2 was found to be inhibited by TA, with an I_{50} value of 13 μM[27]. TA was reported to inhibit 12-O-tetradecanoyl phorbol 13-acetate (TPA)-induced NO generation in rat hepatocyte. It was also found that TA was effective in inhibiting NO formation when added 2 h following TPA addition. More than 90% inhibition in NO release was observed in the presence of a 500 μM concentration of TA, which suggesting to be of use in reducing chronic inflammation and in restoring depleted antioxidant machinery[28].

In our study, consistent with most of the other studies, we found that within the concentrations of 0.1-150 μM TA had an antioxidant capacity against ABTS radical and within the range of 1-10 μM the antioxidant activity of TA was found to be significantly higher than the antioxidant activity of trolox using TEAC assay.

TA inhibited B[a]P metabolites binding to calf thymus DNA by 40% at 40 mM, and B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE): deoxyguanosine adduct formation in calf thymus DNA by 12 to 54% at 10 to 40 mM[29]. Fedeli *et al*[30]*.* investigated the effect of TA on (*Salmo irideus*) erythrocytes using single-cell gel electrophoresis technique and they found that below 100 μM TA did not induce DNA damage but above 100 μM TA induced DNA damage as H_2O_2 did. Labieniec and Gabryelak[31] reported that in B14 Chinese hamster cells, TA in higher concentrations than 1 μM contributed to the changes in the tested molecules and caused protein and DNA damage, however at 1 μM and 5 μM of TA protected DNA damage induced by Cu(II) and H2O2. It was suggested that the most effective DNA repair occurs 1h after the removal of 15, 30, 60 μM of TA in the cells. Wu *et al*[32]*.* reported that TA exhibited slight DNA damage at 100 μg/ml, however TA and its related compounds decreased the DNA strand breaks induced by 3-amino-1-methyl-5H-pyrido (4,3-b) indole (Trp-P-2), 2-amino-1-methyl-6-phenylimadazo (4,5-b) pyridine (PhIP) or H_2O_2 at 0.1-10 μg/ml and TA reduced the levels of oxidised purines in human lymphocytes induced by H_2O_2 at 10 μg/ml. They suggested that TA might act as prooxidant effect at higher doses, however it could enhance lymphocytes resistance towards DNA strand breaks induced by food mutagens or H_2O_2 *in vitro*. In our study, at the concentrations of 0.1-100 μM, TA alone did not induce DNA damage and also significantly decreased oxidative DNA damage induced by 0.05 mM H_2O_2 in human lymphocytes. However we did not determined the prooxidant effect of TA at the concentrations studied.

Within the concentration of 0.1-100 μM TA alone seemed to be significantly increased the level of Fpg sensitive sites suggesting that the increase in DNA damage is the result of oxidative purine base damage, however TA treatment at all the concentrations studied significantly reduced the Fpg sensitive sites induced by H_2O_2 in a dose dependent.

Conclusions

Our results compatible with most of the studies have suggested that TA should not consume at high doses. However TA might have protective effects on H_2O_2-induced oxidative DNA strand breakage. There is much evidence pertaining to the wide beneficial health effects of tannins. It seems that our results highlight the potential benefit of TA as a dietary supplement and a natural antioxidant. But our results concern only *in vitro* experiments with human lymphocytes and the full effects of tannins are not be known. Further studies are needed to clarify the mechanisms involved in protective effects of TA.

Acknowledgements

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Summary

Phenolic substances in natural products play an important role in protecting the organism against harmful effects of reactive oxygen species. Tannic acid (TA), water-soluble polyphenol, is present in many plant beverages and foods, such as herbal teas, beer, walnut, hazelnut, and berries. Tannins have been reported to exert some physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, cause liver necrosis, and modulate immunoresponses. It acts as an antioxidant or a prooxidant, depending on the concentrations of TA and the number and position of hydroxyl groups on TA. It has been suggested that tannins might be carcinogenic, as the incidences of certain cancers, such as esophageal cancer, are related to consumption of tannins-rich foods. In the present study, the antioxidant capacity of TA was determined by the trolox equivalent antioxidant capacity assay and the effect of TA on DNA damage induced by H_2O_2

in human lymphocytes were investigated by the standard comet assay and the formamidopyrimidine-DNA-glycoslase (Fpg) modified comet assay. Our study showed that above the concentrations of 0.5 μM TA showed significant antioxidant capacity. It was determined that at all the concentrations studied (0.1-100 μM), TA alone did not cause DNA damage and even significantly reduced oxidative DNA damage induced by H_2O_2. It was also determined that within the concentration of 0.1-100 μM TA alone did not induce Fpg sensitive sites indicating the increased oxidized purine base levels, but TA treatment at all the concentrations studied significantly reduced the Fpg sensitive sites induced by H_2O_2 in a dose dependent. In conclusion, it seems that TA exerts a protective effect on oxidative DNA damage in human lymphocytes *in vitro*. In appropriate levels as a dietary supplement, TA may be used as a natural antioxidant however it is necessary to perform the further *in vivo* and *in vitro* studies to clarify the potential effects of TA on human health.

Key Words: Tannic acid, Hydrogen peroxide, Single cell gel electrophoresis, Comet assay, TEAC (Trolox equivalent antioxidant capacity) assay

Özet

Tannik Asitin İnsan Lenfositlerinde DNA Hasarı Üzerine Etkisi

Doğal ürünlerde bulunan fenolik maddeler reaktif oksijen türevlerinin zararlı etkilerine karşı organizmayı korumada önemli bir role sahiptir. Suda çözünen polifenolik tannik asit (TA) bitkisel çaylar, bira, ceviz, fındık ve üzümsü meyveler gibi pek çok bitkisel içecek ve gıdalarda bulunmaktadır. Tanenlerin kan pıhtılaşmasını hızlandırdığı, kan basıncını azalttığı, serum lipid düzeyini düşürdüğü, karaciğer nekrozuna neden olduğu ve immün yanıtları düzenlediği gibi bazı fizyolojik etkileri bildirilmektedir. TA konsantrasyonuna ve yapısında bulunan hidroksil grupların sayı ve bulunduğu yere göre antioksidan veya prooksidan olarak rol almaktadır. Ancak, TA'ca zengin gıdaların tüketiminin özofagus kanseri gibi belli kanser sıklıkları ile ilişkili olması nedeniyle TA'nın karsinojenik olabileceği de ileri sürülmektedir. Bu çalışmada TA'nın antioksidan kapasitesi troloks eşdeğer antioksidan kapasite deneyi ile ve insan lenfositlerinde H_2O_2 ile indüklenen DNA hasarı üzerine TA'nın etkisi standart comet yöntemi ve formamidoprimidin-DNA-glikosilaz (Fpg) ile adapte edilmiş comet yöntemi ile araştırılmıştır. Sonuçlarımız 0.5 μM konsantrasyon üzerinde TA'nın anlamlı olarak antioksidan kapasiteye sahip olduğunu göstermiştir. Çalışılan tüm konsantrasyonlarında (0.1-100 μM) TA'nın tek başına DNA hasarına neden olmadığı ve hatta H_2O_2 ile indüklenen oksidatif DNA hasarını anlamlı olarak azalttığı saptanmıştır. Ayrıca

0.1-100 μM konsantrasyon aralığında tek başına TA'nın artmış okside pürin baz düzeylerinin göstergesi olan Fpg duyarlı bölgeleri indüklemediği, ancak TA'nın çalışılan tüm konsantrasyonlarında doz bağımlı olarak H_2O_2 ile indüklenen Fpg duyarlı bölgeleri anlamlı olarak azalttığı saptanmıştır. Sonuç olarak, TA'nın insan lenfositlerinde oksidatif DNA hasarı üzerine koruyucu bir etkiye sahip olduğu *in vitro* olarak görülmektedir. Diyetsel destek olarak uygun düzeylerde tannik asit doğal bir antioksidan olarak kullanılabilir, fakat tannik asitin insan sağlığı üzerine potansiyel etkilerini aydınlatmak için daha ileri *in vivo* ve *in vitro* çalışmalara gerek vardır.

Anahtar Kelimeler: Tannik asit, Hidrojen peroksit, Tek hücre jel elektroforez, Comet yöntemi, TEAK (Troloks eşdeğer antioksidan kapasite) yöntemi

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