Oxidants and Antioxidants in Medical Science
www.oamsjournal.com
DOI: 10.5455/oams.020315.or.081

Original Article

Protective response of gallic acid from oxidative nuclear and mitochondrial DNA damage in HeLa cells

Ozlem Erol[1], Nazli Arda[2], Gunhan Erdem[3]

[1]Department of Biology, Institute of Natural and Applied Sciences, Canakkale Onsekiz Mart University, Canakkale, Turkey, [2]Department of Molecular Biology and Genetics, Faculty of Science, Istanbul University, İstanbul, Turkey, [3]Faculty of Health Sciences, Girne American University, Kyrenia, Turkish Republic of Northern Cyprus

Address for correspondence:
Ozlem Erol, Institute of Natural and Applied Sciences, Canakkale Onsekiz Mart University, Terzioglu Campus, 17020, Canakkale, Turkey.
E-mail: ozlemerolbio@gmail.com

Received: November 12, 2014
Accepted: April 14, 2015
Publishing: April 14, 2015

ABSTRACT

Objective: Oxidative DNA damages occur in the cells constantly exposed to reactive oxygen species that can originate from normal metabolic processes and from environmental agents. Accumulation of oxidative DNA damages has been observed in several pathologies, such as aging, carcinogenesis and degenerative diseases. In this study the hypothesis that gallic acid (GA), one of the most distributed phenolics in plants, could prevent the H_2O_2-induced both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage was investigated. **Materials and Methods:** The cells were pretreated with GA (28 μg/ml) for 4 h before the induction of oxidative stress by H_2O_2 (300 μM) exposure for 1 h. DNA damage was assessed in the mtDNA and two nuclear regions using quantitative polymerase chain reaction (qPCR) assay. **Results:** Pretreatment with GA significantly reduced both nDNA and mtDNA damages occurred with H_2O_2 exposure. **Conclusion:** The results clearly demonstrate that GA has a protective effect against oxidative damage for both nDNA and mtDNA in HeLa cells. GA is most likely to act as an antimutagenic/anticarcinogenic agent through the protection of the genome against the damaging effect of chronic oxidative stress.

KEY WORDS: DNA damage, gallic acid, HeLa, quantitative polymerase chain reaction

INTRODUCTION

Oxidative stress imposed by reactive oxygen species (ROS) plays a crucial role in aging as well as in the development of several diseases. ROS can originate from several internal and external sources, such as normal metabolism, dietary intake of oxidative substances, or cigarette smoking in humans [1]. ROS, mainly in radical forms, induce structural damages in all biomolecules and consequently impair cellular processes [2].

The cells protect themselves from the harmful effect of ROS by various antioxidant defense systems, using several enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic antioxidants, such as glutathione, tocopherols, etc. The diet including antioxidants is very important for the inhibition of oxidative stress [2]. However, these antioxidant defense mechanisms cannot completely cope with oxidative stress and eventually oxidatively damaged molecules can accumulate in the cells. Especially, the accumulation of oxidative DNA damages is announced to play a significant role in the aging process and the progression of age-related diseases [1,3].

Gallic acid (GA) (3,4,5-trihydroxybenzoic acid) is found in many plants, especially in tea leaves and grape seeds [4,5]. The animal and cell culture studies have shown that the GA has anti-carcinogenic and anti-genotoxic activity [6-10]. It is suggested that GA shows the cytotoxicity against some cancer cells [5,11,12], but no cytotoxicity against normal lymphocyte, lung fibroblast (V79-4), and leukemia (K562) cells [7,9,13]. It is shown that GA has a protective effect against cellular DNA damage using alkaline agarose (COMET) assay [7-9]. In our former study, we found that GA is able to inhibit the ROS generation and to prevent HeLa cells against apoptotic cell

death under oxidative stress condition [14]. Similar results indicate that GA has protective effects against apoptosis and several oxidative stress markers such as ROS generation, lipid peroxidation, glutathione depletion and protein carbonyl content *in vitro* and *in vivo* [15-18]. Thus, GA provided by the diet can be regarded as an important contributor in the prevention of the cells against aging, degenerative diseases, and cancer.

Based on the abovementioned facts, the goal of the present study was to find out whether GA could protect nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) damage in HeLa cells under H_2O_2-induced oxidative stress.

MATERIALS AND METHODS

Cell Culture

Human HeLa cervical carcinoma cells were grown in Eagle's Minimum Essential Medium with Earle's saline (EMEM), supplemented with an antibiotic-antimycotic mixture (penicillin 100 U/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml), and 10% (v/v) heat-inactivated fetal bovine serum. The cells were seeded at the concentration of 10^5 cells per milliliter and maintained at 37°C in an atmosphere with 5% CO_2. GA, purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in dimethyl sulfoxide (DMSO; 1 mg/ml) and added to the growth medium, at a final concentration not exceeding 0.5% (v/v), since DMSO inhibits cell growth above this concentration (data not shown).

Cytotoxicity Test

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay was used as described previously [19] in order to estimate cell viability. This method was based on the ability of viable cells to reduce the substrate, MTT and to form a formazan product. The cells (10^5 cells/ml) were seeded onto the well, containing 200 μl EMEM in a 96-well plate and then treated with GA (1-200 μg/ml) for the last 4 h of a total incubation time of 72 h. For the determination of cytotoxicity of H_2O_2; HeLa cells treated with H_2O_2 (0.005-2 mM) for the last 1 h of a total incubation time of 72 h. At the end of the treatment period, the culture medium was removed and the cells were washed with phosphate-buffered saline (PBS), and 30 μl of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 4 h. After the formation of formazan crystals, 200 μl of DMSO was added to each well and the resulting optical density was measured by a microplate reader (μQuant, BioTek Instruments, Winooski, VT, USA) at 570 nm and 690 nm (reference) wavelengths. The cell viability was calculated as a percentage of viable cells in the experimental group *vs* untreated control group using the following formula:

$$\text{Cell viability (\%)} = (\text{Optical density}_{exp}/\text{Optical density}_{control}) \times 100$$

The EC_{50} dose on HeLa cells was calculated by GraphPad Prism software from a graph of cell viability of GA and H_2O_2. The non-toxic dose of the plant extract (28 μg/ml) was used for further experiments.

GA Pre-treatment and Induction of Oxidative Stress

The cells (10^5 cells/ml) were seeded into a 25 cm^2 flask containing 7.5 ml EMEM and maintained at 37°C in an atmosphere with 5% CO_2 for 68 h. After the incubation time of 68 h, HeLa cells were treated for 4 h with 28 μg/ml of GA for the GA-treated group or with EMEM only for the untreated control. Oxidative stress was generated by (300 μM) H_2O_2. Following the precise implementation of the test materials, the cells were washed with PBS followed by incubation with H_2O_2 for 1 h. Phenol red-free modified Hank's Balanced Salt Solution was used as a diluent for H_2O_2 and as a blank in the assays. The H_2O_2 concentration was checked by the absorbance at 240 nm [20].

DNA Isolation and Quantitative Polymerase Chain Reaction (qPCR)

High molecular weight total cellular DNA was isolated with the GenElute™ Mammalian Genomic DNA isolation kit (Sigma) according to the manufacturer's protocol. The concentration of total cellular DNA was determined using the Quant-iT™ dsDNA High-Sensitivity Assay Kit in a Qubit™ fluorometer (Invitrogen, Paisley, UK).

Quantitative PCR was carried out as described earlier [21]. Three different genomic regions were analyzed for oxidative damages. The specific oligonucleotide primers for a 2082 bp fragment of the nuclear *APEX1* gene (transcribed, GenBank ID: X66133), a 2334 bp fragment of the nuclear B-globin gene cluster (non-transcribed, GenBank ID: NG_000007) and a 2232 bp fragment of the mtDNA (GenBank ID: AF347015) in HeLa cells were used in the PCR reactions [21]. All the PCR reactions were performed in a Techne TC-3000 Thermal Cycler (Bibby Scientific; Staffordshire, UK).

A quantitative control using the half of the concentration of control template DNA was included in each set of PCR reaction. The small fragments (161 and 180 bp) of the mitochondrial and nuclear region were also amplified as internal controls, respectively. The internal controls were used to normalize the results obtained from the large fragments and to monitor the mitochondrial copy number. An aliquot of each PCR product was checked by the electrophoresis on a 2% (w/v) vertical agarose gel at 70 V for 45 min in 1 × TAE buffer (Tris-Acetate-EDTA, pH 8). Final PCR products were quantitated using Quant-iT™ dsDNA High Sensitivity Assay Kit. The average lesion frequency per each fragment was calculated by the Poisson equation [22].

Statistical Analysis

The data were expressed as mean ± standard error of the mean of three independent experiments done in triplicates. All analyses were carried out using GraphPad Prism (version 5.00 for Windows, GraphPad Inc; San Diego, CA, USA) software.

Statistical analyses were performed using one-way Analysis of Variance followed by Dunnett's multiple comparison tests.

RESULTS

The purpose of the present study was to evaluate the possible protective effect of GA on the oxidative damage caused by H_2O_2 in the mitochondrial and nuclear genomes of HeLa cells. The effects of GA and H_2O_2 on HeLa cell viability were investigated by MTT assay, based on the reduction of MTT to a colored formazan product by mitochondrial dehydrogenase, which is active only in living cells. GA decreased the viability of HeLa cells in a dose-dependent manner for 4 h treatment [Figure 1]. Thus, in order to avoid the cytotoxic effects of GA, its concentrations were optimized, and a non-cytotoxic dose (28 μg/ml) was used for further experiments. H_2O_2 has an EC_{50} value of 808 μM in HeLa cells.

In this study, oxidative stress consequently DNA damage in HeLa cells was provoked by H_2O_2. The gene-specific qPCR assay was performed on nuclear regions (*APEX1* and β-globin) and mtDNA for the detection of oxidative damage caused by H_2O_2. This assay was based on the fact that many DNA lesions could block the Taq polymerase and, as a result, the product amplification was decreased [22].

No changes in the steady-state level of both nuclear and mitochondrial DNA damage were detected in the GA treated cells under non-stressed conditions [Figure 2]. The lesion frequencies in transcribed *APEX1* and mtDNA were damaged at a similar level by 300 μM H_2O_2, while β-globin region was exposed to less damage by H_2O_2 [Figure 3] because it is a non-transcribed gene and tightly packaged in nucleosome. There was no statistically significant difference between oxidative mtDNA and nDNA (*APEX1*) damage levels provoked by 300 μM H_2O_2. Pretreatment with GA significantly decreased the damages caused by the oxidative stress in the lesion frequencies in both *APEX1* (nDNA) and mtDNA.

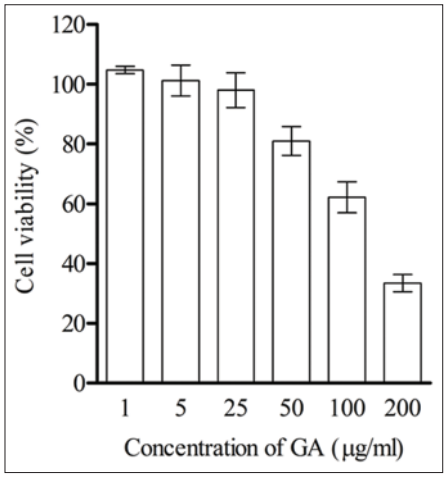

Figure 1: Effect of increasing concentration of gallic acid on cell viability.

DISCUSSION

It is well-known that ROS cause oxidative DNA damage and promote the development of several diseases, especially cancer, whereas plant-rich diets decrease oxidative DNA damage and delay or prevent the development of these diseases [23]. Phenolic antioxidants are ubiquitous in plants and have been considered as protectants against cancer, diabetes, cardiovascular diseases, and diseases related to oxidative stress [10,24]. GA is one of the major phenolic constituents of many plants, such as grape seed and tea and acts as an excellent antioxidant [4,5].

A previous study in cell-free system demonstrates that GA possesses a protective effect against DNA strand breakage induced by nitric oxide, H_2O_2 and the hydroxyl radical

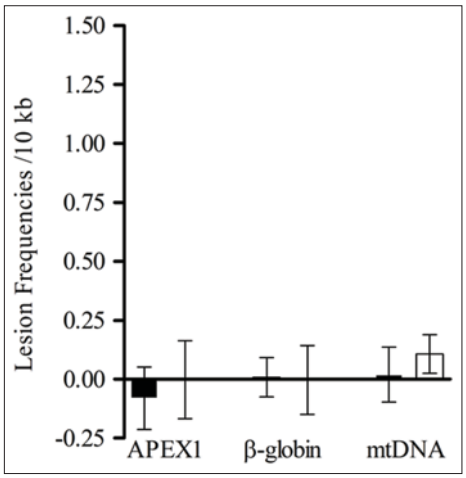

Figure 2: Effect of gallic acid (GA) on the background DNA damage in HeLa cells (black bars, GA-untreated cells; white bars, GA-pretreated cells).

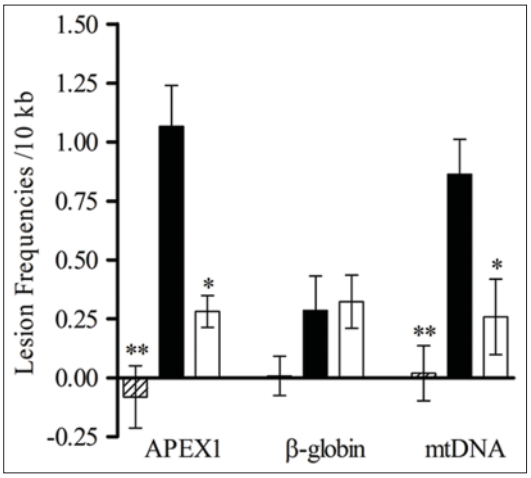

Figure 3: DNA lesion frequencies in HeLa cells. Control cells were untreated with neither gallic acid (GA) nor H_2O_2 (raked bars); GA-untreated (black bars) and GA-pretreated cells (white bars) were oxidatively stressed by 300 μM H_2O_2. *P < 0.05 and **P < 0.001 compared to treatment with H_2O_2

(•OH) [25]. However, GA induces DNA damage in the presence of metal ions such as iron and copper, under experimental conditions [26]. Metal-mediated DNA damage caused by GA is mainly due to •OH generated via the Fenton reaction [26]. However, GA can reduce the levels of oxidative stress markers such as lipid peroxidation and glutathione depletion [15,27].

The results of our previous study indicated that the pretreatment with GA for 4 h caused a significant reduction in the steady-state level of ROS. Moreover, the ROS level of HeLa cells under oxidative stress, which pretreated with GA for 4 h were less than basal ROS level [14]. An inhibitory effect on apoptotic cell death of GA in human peripheral blood lymphocytes, HeLa cells and rat was reported [14,18,28]. This effect seemed to be due to its direct action in the scavenging of free radicals [18,29].

The purpose of the present study was to evaluate the possible protective effect of GA on nDNA and/or mtDNA damage in HeLa cells, as a model. We demonstrated that the pretreatment of HeLa cells with GA could prevent nDNA and mtDNA damage induced by 300 μM H_2O_2. The steady-state levels of nDNA and mtDNA damage were not changed upon the treatment of the cells with non-cytotoxic concentration of GA (28 μg/ml). This result indicated that GA at this concentration did not damage to DNA. These data were in agreement with the previous reports of the decreased total DNA damage in various cells by GA pre-treatment before exposure to H_2O_2 [7,9,27]. Similarly, Gandhi and Nair [8] observed that GA was able to completely inhibit DNA strand breaks caused by gamma radiation in human lymphocytes. Animal studies also demonstrated that the administration of GA prior to the whole body gamma radiation exposure reduced the lipid peroxidation and cellular DNA damage [8]. Another study showed that topical application and oral feeding with GA had a protective effect against tumor growth and progression in mice [6,10]. Other study indicated that GA treatment had the reducing effect on thiobarbituric acid reactive substances and protein carbonyl content in brain tissue of rats acutely exposed to lead [16]. In addition, GA had neuroprotective activity against 6-hydroxydopamine-induced oxidative stress via enhancement of the cerebral antioxidant defense in rats [17].

The data of the present study confirmed the previous observations on the relationship between the inhibition of intracellular ROS and the antioxidant activity of GA. The effects of GA in different cells as antioxidant defenses restoration and inhibition of intracellular ROS production were reported [14,15]. Accordingly, Abdelwahed *et al.* [9] suggested that decreased DNA damage was associated with a direct effect on DNA repair enzymes through modulating their gene expression.

Thousands of DNA damages per day in each cell in human occur by the endogenous and exogenous stresses [30] and if the cell do not have effective DNA repair systems and/or antioxidant defenses, this DNA damage accumulates and may result in age-related disorders, especially cancer [3]. In addition, it was determined that a mutation in a gene important for DNA repair predisposes to several cancers, such as colon, breast, and skin [3]. DNA damage in tumor suppressor genes and oncogenes may also be responsible for the progression of cancer. Our results suggest that GA could protect against this accumulation by the inhibition of oxidative DNA damage.

The present study revealed, for the first time, that GA is able to protect both nuclear and mitochondrial genome from oxidative stress caused by H_2O_2. In conclusion, GA is most likely to act as an anti-mutagenic/anti-carcinogenic agent through the protection of the genome against the damaging effect of chronic oxidative stress. The concentration of GA used in this study might be present in the gastrointestinal track after the consumption of GA [27,31]; therefore, our findings support a possible physiologically relevant protective effect of GA against ROS in the intestine [18]. Furthermore, the DNA protection effect of GA may also contribute to the beneficial effects of many plant extracts. These findings underscore the importance of further studies in order to define the possible beneficial outcomes of the dietary use of GA.

REFERENCES

1. Bohr VA. Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic Biol Med 2002;32:804-12.
2. Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radic Res 1996;25:57-74.
3. Hasty P. The impact of DNA damage, genetic mutation and cellular responses on cancer prevention, longevity and aging: observations in humans and mice. Mech Ageing Dev 2005;126:71-7.
4. Kuhr S, Engelhardt UH. Determination of flavanols, the ogallin, gallic acid and caffeine in tea using HPLC. Z Lebensm Unters Forsch 1991;192:898-904.
5. Veluri R, Singh RP, Liu Z, Thompson JA, Agarwal R, Agarwal C. Fractionation of grape seed extract and identification of gallic acid as one of the major active constituents causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. Carcinogenesis 2006;27:1445-53.
6. Soleas GJ, Grass L, Josephy PD, Goldberg DM, Diamandis EP. A comparison of the anticarcinogenic properties of four red wine polyphenols. Clin Biochem 2002;35:119-24.
7. Yen GC, Duh PD, Tsai HL. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. Food Chem 2002;79:307-13.
8. Gandhi NM, Nair CK. Protection of DNA and membrane from gamma radiation induced damage by gallic acid. Mol Cell Biochem 2005;278:111-7.
9. Abdelwahed A, Bouhlel I, Skandrani I, Valenti K, Kadri M, Guiraud P, *et al*. Study of antimutagenic and antioxidant activities of gallic acid and 1,2,3,4,6-pentagalloylglucose from *Pistacia lentiscus* conformation by microarray expression profiling. Chem Biol Interact 2007;165:1-13.
10. Raina K, Rajamanickam S, Deep G, Singh M, Agarwal R, Agarwal C. Chemopreventive effects of oral gallic acid feeding on tumor growth and progression in TRAMP mice. Mol Cancer Ther 2008;7:1258-67.
11. Inoue M, Suzuki R, Sakaguchi N, Li Z, Takeda T, Ogihara Y, *et al*. Selective induction of cell death in cancer cells by gallic acid. Biol Pharm Bull 1995;18:1526-30.
12. Sakaguchi N, Inoue M, Ogihara Y. Reactive oxygen species and intracellular Ca2+, common signals for apoptosis induced by gallic acid. Biochem Pharmacol 1998;55:1973-81.
13. Yoo KM, Lee CH, Lee H, Moon B, Leen CY. Relative antioxidant and cytoprotective activities of common herbs. Food Chem 2008;106:929-36.
14. Erol-Dayi Ö, Arda N, Erdem G. Protective effects of olive oil phenolics and gallic acid on hydrogen peroxide-induced apoptosis. Eur J Nutr 2012;51:955-60.
15. Pourahmad J, Eskandari MR, Shakibaei R, Kamalinejad M. A search for hepatoprotective activity of fruit extract of *Mangifera indica* L. against oxidative stress cytotoxicity. Plant Foods Hum Nutr 2010;65:83-9.

16. Reckziegel P, Dias VT, Benvegnú D, Boufleur N, Silva Barcelos RC, Segat HJ, *et al.* Locomotor damage and brain oxidative stress induced by lead exposure are attenuated by gallic acid treatment. Toxicol Lett 2011;203:74-81.
17. Mansouri MT, Farbood Y, Sameri MJ, Sarkaki A, Naghizadeh B, Rafeirad M. Neuroprotective effects of oral gallic acid against oxidative stress induced by 6-hydroxydopamine in rats. Food Chem 2013;138:1028-33.
18. Pal C, Bindu S, Dey S, Alam A, Goyal M, Iqbal MS, *et al.* Gallic acid prevents nonsteroidal anti-inflammatory drug-induced gastropathy in rat by blocking oxidative stress and apoptosis. Free Radic Biol Med 2010;49:258-67.
19. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
20. Aebi H. Catalase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. New York: Academic Press; 1974. p. 673-84.
21. Erol Ö, Arda N, Erdem G. Phenols of virgin olive oil protects nuclear DNA against oxidative damage in HeLa cells. Food Chem Toxicol 2012;50:3475-9.
22. Kovalenko OA, Santos JH. Analysis of oxidative damage by gene-specific quantitative PCR. Curr Protoc Hum Genet 2009;62:19.1.1-19.1.13.
23. Halliwell B. Effect of diet on cancer development: is oxidative DNA damage a biomarker? Free Radic Biol Med 2002;32:968-74.
24. Liu Z, Sun L, Zhu L, Jia X, Li X, Jia H, *et al.* Hydroxytyrosol protects retinal pigment epithelial cells from acrolein-induced oxidative stress and mitochondrial dysfunction. J Neurochem 2007;103:2690-700.
25. Ohshima H, Yoshie Y, Auriol S, Gilibert I. Antioxidant and pro-oxidant actions of flavonoids: effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. Free Radic Biol Med 1998;25:1057-65.
26. Kobayashi H, Oikawa S, Hirakawa K, Kawanishi S. Metal-mediated oxidative damage to cellular and isolated DNA by gallic acid, a metabolite of antioxidant propyl gallate. Mutat Res 2004;558:111-20.
27. Ferk F, Chakraborty A, Jäger W, Kundi M, Bichler J, Mišík M, *et al.* Potent protection of gallic acid against DNA oxidation: results of human and animal experiments. Mutat Res 2011;715:61-71.
28. Sohi KK, Mittal N, Hundal MK, Khanduja KL. Gallic acid, an antioxidant, exhibits antiapoptotic potential in normal human lymphocytes: A Bcl-2 independent mechanism. J Nutr Sci Vitaminol (Tokyo) 2003;49:221-7.
29. Vijaya Padma V, Sowmya P, Arun Felix T, Baskaran R, Poornima P. Protective effect of gallic acid against lindane induced toxicity in experimental rats. Food Chem Toxicol 2011;49:991-8.
30. Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993;362:709-15.
31. Konishi Y, Hitomi Y, Yoshioka E. Intestinal absorption of p-coumaric and gallic acids in rats after oral administration. J Agric Food Chem 2004;52:2527-32.

Source of Support: Nil, Conflict of Interest: None declared.