Potent protection of gallic acid against DNA oxidation: Results of human and animal experiments

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Gallic acid (3,4,5-trihydroxybenzoic acid, GA) is a constituent of plant derived foods, beverages and herbal remedies. We investigated its DNA protective properties in a placebo controlled human intervention trial in single cell gel electrophoresis experiments. Supplementation of drinking water with GA (12.8 mg/person/d) for three days led to a significant reduction of DNA migration attributable to oxidised pyrimidines (endonuclease III sensitive sites) and oxidised purines (formamidopyrimidine glycosylase sensitive sites) in lymphocytes of healthy individuals by 75% and 64% respectively. Also DNA damage caused by treatment of the cells with reactive oxygen species (ROS) was reduced after GA consumption (by 41%). These effects were paralleled by an increase of the activities of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and glutathione-S-transferase–α) and a decrease of intracellular ROS concentrations in lymphocytes, while no alterations of the total antioxidant capacity (TAC), of malondialdehyde levels in serum and of the urinary excretion of isoprostanes were found. Experiments with rats showed that GA reduces oxidatively damaged DNA in lymphocytes, liver, colon and lungs and protects these organs against γ-irradiation-induced strand breaks and formation of oxidatively damaged DNA-bases. Furthermore, the number of radiation-induced preeponicotic hepatic foci was decreased by 43% after oral administration of the phenolic. Since we did not find alterations of the TAC in plasma and lipid peroxidation of cell membranes but intracellular effects it is likely that the antioxidant properties of GA seen in vivo are not due to direct scavenging of radicals but rather to indirect mechanisms (e.g. protection against ROS via activation of transcription factors). As the amount of GA used in the intervention trial is similar to the daily intake in Middle Europe (18 mg/person/day), our findings indicate that it may contribute to prevention of formation of oxidatively damaged DNA in humans.

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1. Introduction

Numerous investigations indicate that reactive oxygen species (ROS) are implicated in the development/progression of cancer and possibly involved in the aetiology of other human diseases, (for reviews see [1,2]). Over the last decade, numerous antioxidants have been discovered in the diet, but evidence for their protective properties is in many cases restricted to results of in vitro experiments [3].

Recently, we found in a human trial that sumach (Rhus coriaria) which is used as a spice [4], prevents formation of oxidatively damaged DNA in lymphocytes. In subsequent experiments with rats, we detected pronounced DNA protective effects in internal organs and results of in vitro experiments with lymphocytes indicated that gallic acid (GA) may be the active compound [5].

GA is contained in a variety of human foods of plant origin such as blueberries and strawberries [6], mangos [7], grape seeds [8] and carob [9]. Also beverages such as red wine [10], teas [11–13] and coffee [14] contain high amounts of GA and the phenolic acid was-

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also postulated to account for the beneficial effects of several herbal remedies used in traditional medicine [15,16].

At present, evidence for the DNA protective properties of GA is restricted to results of in vitro experiments with different cell lines and human blood cells [17–21]. However, it was found in animal studies, in which high doses were administered, that it induces antioxidant enzymes [22], protects the liver against cytotoxic effects [23] and prevents radiation-induced lipid peroxidation (LP) [24].

Aim of the present study was the investigation of the prevention of DNA damage by GA in humans. We conducted a placebo controlled intervention trial in which the participants consumed a low dose of GA. DNA damage was measured in single cell gel electrophoresis (SCGE) assays which are based on the determination of DNA migration in an electric field and are increasingly used in human studies to investigate the effects of dietary factors (for reviews see [25–27]). Subsequently, the effects of GA on formation of strand breaks and oxidised DNA bases were measured; furthermore, we monitored the impact of GA on the sensitivity of lymphocytes towards induction of DNA damage by ROS [28] by hydrogen peroxide treatment. To elucidate the molecular mechanisms of the DNA protective effect, we monitored alterations of the activities of antioxidant enzymes (glutathione peroxidase (GPX), superoxide dismutase (SOD) and glutathione S-transferases (GSTD)) in plasma and a number of biochemical parameters that reflect the antioxidant status (total antioxidant capacity (TAC) and LP markers such as MDA in plasma and isoprostane levels in urine.

To find out if GA is also protective against DNA damage in internal organs, we conducted experiments with rats in which the animals were exposed to γ-radiation which causes DNA strand breaks but also formation of oxidatively damaged DNA bases due to formation of hydroxyl and superoxide radicals as a consequence of cleavage of water molecules [29]. These radicals are also formed from hydrogen peroxide which was used in the human study. Radiation experiments offer the advantage that they reflect the in vivo situation; furthermore, they enable to draw conclusions if prevention of DNA damage is associated with protection against cancer. We monitored the impact of GA on prevention of DNA strand breaks and formation of oxidised bases in SCGE experiments in lymphocytes, lungs, liver, colon and brain under identical experimental conditions as in the human intervention trial. To elucidate, if the prevention of DNA damage is linked to protection against cancer, additional experiments were carried out in which we monitored the impact of GA on formation of enzyme altered (GST-p+) foci in the liver which are regarded as precursors of liver tumours [30].

2. Materials and methods

Acetic acid, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals (ABTS), acetonitrile, inorganic salts, bovine serum albumin fraction V (BSA), 1-chloro-2,4-dinitrobenzene (DNCB), diamobenzidine, dimethyl sulfoxide (DMSO), epinephrine, ethidium bromide, gallic acid (GA, CAS No. 149-81-7, purity 99%), Histopaque-1077, RPMI 1640, methanol, NaOH, Trizma base, Triton X-100, trypan blue and 2-thiobarbituric acid (TBA) were purchased from Sigma–Aldrich (Glostrup, Denmark). Formamidopyrimidine glycoseylase and endonuclease III (endo III) were a gift of AR Collins (University of Oslo, Norway). The protein assay came from BIO-RAD® (Munich, Germany). HEPKTR™-Alpha, HEPKTR™-Pi and Anti-GST-p were purchased from Biotin International Ltd. (Dublin, Ireland). 7-Dichloro-fluorescin (DCFH), 2,7'-dichlorofluorescin diacetate (DCFH-DA), glutathione disulfide (GSSG), glutathione reductase, hydrogen peroxide, myoglobin, reduced glutathione (GSH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Fluka Chemicals (Buchs, Switzerland). Anti-rabbit immunoglobulin (rabbit polyclonal IgG), biotinylated goat anti-rabbit IgG, horseradish peroxide-anti-peroxidase and strepavidin complex came from Dakopatts (Glostrup, Denmark). The urinary 8-epi-prostaglandin-F2α, Kit (catalog no. 21048) was from Oxis, International Inc. (Portland, USA.). PerCP labeled anti-CD-45 (pan-leukocytic marker) and APC labeled anti-CD-14 (monocyte/macrophage specific) antibodies were purchased from BD Pharmingen (San Jose, CA, USA).

2.2. Design of the human studies

The studies were approved by the Ethics Committee of the Medical University of Vienna and informed consent was obtained from all participants. The participants were recruited from three medical volunteers. Sixteen healthy volunteers (eight men: mean age: 34 ± 5 y, b.w.: 68 ± 3 kg, BMI: 21.8 ± 3.0 and eight women: mean age: 32 ± 5 y, b.w.: 60 ± 4 kg, BMI: 20.3 ± 4.1), who were all non-smokers, non-vegetarians and non-users of medications and dietary supplements participated in the study and were randomly allocated either into the placebo group or into the GA group (each group consisted of 4 male and 4 female participants). Before the start of the study all of them filled out a lifestyle questionnaire. All participants consumed seven days before and during the intervention (3 days) a regular diet without specific food products (including GA rich fruits such as mangos, apricots, grapes, berries and apples) and antioxidant rich food items (kiwis, citrus fruits, carrots, tomatoes, spinach, cabbage, onions and garlic) as well as spices (curcumin, ginger, chilies). Furthermore, they did not consume coffee, tea, wine and vitamin supplemented drinks and were asked not to perform excessive physical exercises, which may cause DNA damage [31].

During the intervention, each participant consumed 0.2 mg/kg GA/d dissolved in 500 mL drinking water over a period of 3 consecutive days (average intake 12.8 mg/person per day). Every day, aqueous GA solution was prepared fresh at 9 a.m. and distributed to the participants who consumed it within a period of 2 h. The intervention time was selected on the basis of results of an earlier human trial with oncum [4]. The amount of GA corresponds to the ADI defined by FAO/WHO for gallates [32]. The participants of the placebo group drank an identical amount of no-supplemented water. Blood samples (2 x 10 mL) were collected in heparinised tubes (BD Vacutainer, Plymouth, UK) immediately before (T0) and after (T1) the intervention.

After the first trial we conducted a further study with an identical design (but without a placebo) with six participants (three males and three females) which had been also involved in the first study. At the end of the intervention, blood and plasma samples were collected for the analysis of DCFH.

2.3. Determination of GA in plasma

An additional intervention experiment was conducted in which we measured the concentrations in plasma of three participants. Three participants were consumed a GA solution (0.2 mg/kg) over three days. On the third day, blood samples were collected after different time intervals (1, 2 and 4 h) in heparinised tubes. After centrifugation (760 x g, 10 min), the plasma samples were aliquoted and stored at –80°C.

The concentrations of GA in blood and water were measured using a Dionex “UltiMate 3000” system (Dionex Corp., Sunnyvale, CA). The column oven was set at 35 °C and the UV-Detector at 270 nm. Briefly, after addition of 500 μL of acetonitril to 250 μL of serum, the samples were centrifuged (5000 x g for 5 min at 4 °C) and 400 μL of the samples were injected into the HPLC column. Separation of GA was carried out using a Hypersil BDS-C18 column (5 μm, 250 mm x 4.6 mm I.D., Astmoor, England) preceded by a Hypersil BDS-C18 pre-column (5 μm, 10 mm x 4.6 mm I.D.) at a flow rate of 1.0 mL/min. The mobile phase consisted of a continuous linear gradient, mixed from 0.1% acetic acid (mobile phase A) and methanol (mobile phase B). The gradient ranged from 0/8 (0 min) to 80/20 at 20 min and decreased to 0/0 at 21 min. The columns were allowed to re-equilibrate for 9 min between runs. Linear calibration curves were performed from the peak areas of GA to the external standard by spiking drug-free human serum with standard solutions of GA (final concentrations ranging from 0.01 μg to 10.0 μg/mL). The detection limit of GA was 0.057 μg/mL with coefficients of accuracy and precision <0%. 2.4. SCGE assays with human and rat lymphocytes

The experiments were carried out according to the guidelines for SCGE experiments [33,34].

The in vitro experiments (Fig. 1) were carried out as described in the article of Bichler et al. [35]. Briefly, lymphocytes were isolated from the blood of a healthy male donor, washed with PBS (pH 7.4) and transferred to RPMI medium. The cells were treated in Eppendorf vials for 60 min with different concentrations of GA (0.015–0.15 μg/mL). To some of the cultures H2O2 (75 μM) was added for 5 min at the end of the exposure period. To terminate the treatment, the cells were centrifuged and washed twice with PBS (pH 7.4). The viability of the cells was monitored with trypan blue dye exclusion technique and was in all cultures ≥80%. For each experimental point, three cultures were made in parallel and from each, 50 cells were analysed for comet formation (see below).

The SCGE protocol which was used in the intervention trial is described in detail in earlier publications [35,36]. All participants in the placebo and in the GA group started and ended the intervention at the same time and the blood was collected at same time of the day 2h after the last consumption of the GA solution.

The assays were performed with fresh lymphocytes from all participants within 2 h after blood collection. To assess the impact of day-to-day variations, experiments
with deep frozen lymphocytes from one donor were included in each experiment. Sampling and analyses were carried out at the same laboratory.

The lymphocytes (1.5 × 10⁶ per tube) were cultured in RPMI 1640 in Eppendorf tubes (Eppendorf AG, Hamburg, Germany). Subsequently, the cells were washed and centrifuged (8 min, 110 × g) twice in phosphate-buffered saline (PBS, pH 7.4). Additionally, the cells were mixed with 0.25% LMA and transferred to agarose coated slides (1.0% NMA). After lysis (pH 10.0) and electrophoresis (20 min, 300 mA, 1.0 V/cm) corresponding to 25V, at 4°C, pH > 13), the gels were stained with ethidium bromide (20 µg/ml).

Additionally, experiments were conducted in which intact cells were treated with H2O2 (75 µM, 5 min, on ice). To determine formation of oxidatively damaged DNA bases, nuclei were exposed after lysis to the endonucleases formamidopyrimidine glycosylase (FPG) and endonuclease III (ENDO III) to determine the optimal amounts of the enzymes, calibration experiments were carried with blood cells from a single donor according to the protocol of Collins et al. [37].

After lysis, the slides were washed twice in enzyme reaction buffer (pH 8.0) for 8 min. Subsequently, 50 µl of FPG or ENDO III (1.0 µg/ml) or enzyme buffer alone were added to the nuclei. The incubation time for FPG was 30 min and for ENDO III 45 min at 37°C respectively. After the treatment, electrophoresis was carried out (30 min, 300 mA, 1.0 V/cm, at 4°C, pH > 13) as described by Collins and Dusinska [38]. In all these experiments, we included parallel measurements, in which the nuclei were treated with the enzyme reaction buffers only.

The viability of the cells was determined in each experiment with the trypan blue (0.4%) dye exclusion technique [39] with an improved Neubauer hemocytometer (Paul Marienfeld GmbH, Lauda-Königshofen, Germany). DNA damage was only analysed in cells from samples in which the viability was ≥80%, as acute toxic effects may cause false positive results [40]. From each participant, three slides were prepared on each laboratory and from each slide, 50 cells were evaluated. Tail-length was measured with the computer aided image analysis system developed by Helma and Uhl which is available via the internet [41]. This parameter has been used in a number of earlier studies; according to the guidelines for SCGE assays it is an acceptable endpoint and it is also mentioned in other methodological papers [33,34,42].

Online supplement 1 (OS1) shows pictures of comets in peripheral lymphocytes and the corresponding tail length values.

2.5. Enzyme measurements

The plasma samples were stored at −80°C and transported on dried ice to the laboratories of the partners.

The activity of GPx was monitored spectrophotometrically in plasma according to the protocol of Flohe and Gunzler [43] which is based on the reduction of NADPH. Absorbance (λ 340 nm) was measured with a UV–vis spectrophotometer (Ultraspex 2000, Pharmacia Biotech Ltd., Cambridge, England).

The activity of SOD was determined in plasma. The measurement of the enzyme is based on its ability to inhibit auto-oxidation of epinephrine [44]. This compound oxidizes rapidly at pH 10.4 producing adrenochrome, a pink coloured product that can be detected at 480 nm. Addition of samples containing SOD inhibits the auto-oxidation of epinephrine. The inhibition rate was monitored continuously over 5 min. The amount of enzyme required to produce a 50% inhibition at 30°C was defined as one unit of enzyme activity. The SOD activity was calculated as the U/L in plasma.

Overall GST activity was measured spectrophotometrically (λ 340 nm) in plasma according to Habig et al. [45] with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GST-α and GST-π were determined with commercial kits (Biotrin) by use of a microplate reader (LKB 5060-006, Roma, Italy). Protein contents in plasma were determined spectrophotometrically according to Bradford [46] with the BIO-RAD® Protein Assay. All measurements were carried out in triplicate.

2.6. Determination of malondialdehyde (MDA)

MDA levels were determined in plasma according to the method of Rames et al. [47]. The samples were neutralized after heating (60 min, 100°C) with methanol/NaOH centrifuged (3 min, 3000 rpm) and MDA was measured with HPLC (excitation: λ 532 nm, emission: λ 563 nm, LaChrom Merck Hitachi Chromatography System, Vienna, Austria). Each sample was measured in duplicate.

2.7. Determination of ROS in human peripheral lymphocytes

Intracellular ROS were measured by flow cytometric analysis of 2,7'-dichlorofluorescin diacetate (DCFH-DA) oxidation using 2,7'-dichlorofluorescein diacetate (DCFH-DA), as described previously by Rothe and Valet [48]. Isolated cells were stained with PerCP-labeled anti-CD-45 (pan-leukocytic marker) and APC labeled anti-CD-14 (monocyte/macrophage specific) antibodies for 15 min. Afterwards, DCFH-DA (10 µM) was added to the suspensions in presence and absence of autologous plasma (1:0).

Furthermore, measurements were performed in which the lymphocytes were exposed additionally to 50 µM H2O2 (60 min at 37°C). After treatment, ROS were monitored by multiparametric analysis using a FACS Calibur TM system (Becton-Dickinson, San Jose, CA) with excitation and emission settings of 500 ± 15 and 535 ± 15 nm respectively. Lymphocytes, monocytes and granulocytes were defined by scatter gating strategy (low, moderate and high FSC/SSC) and CD staining (CD45+CD14-, CD45+CD14+ and CD54dim/CD14dim). Background fluorescence was determined in DCFH-delabled cells without treatment. Each sample was measured in duplicate. Data are given as mean fluorescent peak heights in arbitrary units.

2.8. Total antioxidant capacity (TAC)

TAC was analysed in plasma photometrically after iron-induced oxidation according to the method of Miller et al. [49]. The assay is based on the ability of an antioxidant to scavenge 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals (ABTS), which are generated by the peroxidase activity of metmyoglobin in presence of the sample. The measurements were conducted at λ 734 nm (UV–vis Spectrometer Lambda 2, Perkin Elmer, MA, USA). Each measurement was carried out in duplicate.

2.9. Urinary isoprostane levels

8-Epi-Prostaglandin F2α (8-Epi-PGF2α) was determined with a competitive enzyme-linked immunoassay (Biosynex Urinary 8-Epi-Prostaglandin F2α, Oxiris Research, Portland, USA). 24-h urine was collected and the total amount of the urine was recorded. Subsequently, the samples were aliquoted and stored at −80°C.

Before the measurements, the samples were diluted according to their creatinine concentrations which were determined according to the protocol of Huidan and Rapoport [50]. The samples were subsequently mixed with an enhancing reagent (provided by the manufacturer) that prevents interference due to non specific binding. Subsequently, 8-epi-PGF2α conjugated horseradish peroxidase (HR conjugate) was added, which competes with 8-epi-PGF2α for binding to a specific polyclonal antibody coated on the microplates. After incubation (120 min), the plates were washed and substrate was added. Subsequently, the intensity of the color was measured spectrophotometrically (λ 450 nm). The absorbance is inversely proportional to the amount of unconjugated 8-Epi-PGF2α in the sample.

Absorbance of samples and standards was read using a microplate reader (LKB, 5060-006, Roma, Italy). 8-Epi-PGF2α concentrations were calculated on the basis of urinary creatinine concentrations and expressed in ng/mg creatinine. Each measurement was carried out in duplicate.

2.10. Treatment of the animals

The animal studies were approved by the Ethics Committee of the Medical University of Vienna. All experiments were carried out with male Hm-OFa rats (body weight 280 ± 10 g) which were obtained from the breeding facility of the Medical University of Vienna (Himberg, Austria). Before the start of the experiments, the animals were allowed to acclimatize one week and were housed in plastic cages (Macronyl type III, Techniplast GmbH, Hohenpeissenberg, Germany) under standard conditions (24 ± 1°C, humidity 50 ± 5%, 12 h light/dark cycle) and were fed with a standard diet (R/M-H, Sniff, Soest, Germany).

Before the study with GA, the daily water consumption was determined over a period of two weeks (19.7 ± 5.5 ml). On the basis of the results of these experiments, the GA-concentration in the drinking water was established in such a way, that the average daily consumption was similar to the amount given to the participants in the intervention trial. Every day fresh drinking water of animals was supplemented with GA (2.31 mg/L). The control group received pure water.

The stability of GA in the drinking water given to the animals was determined with the HPLC procedure described above. The GA solution was found to be reason-
ably stable at room temperature. After 24 h, the formation of degradation products was 35.6 ± 2.4%. On the basis of these measurements, we calculated that the total daily intake of GA was slightly lower than the dose used in the human intervention, namely 0.165 mg/kg b.w. per animal.

2.11. Determination of DNA-migration in different organs of rats

The experiments were either carried out with un-irradiated animals or after whole body irradiation in a 60Co-source (GAMMACELL 220, Atomic Energy of Canada Ltd.). Per experimental group, 3 animals were randomly assigned. The rats were irradiated (7.74 Gy, 1.0 min; Nuclear Engineering, Austrian Research Centre, Seibersdorf, Austria) after a 72 h-supplementation period of the drinking water with GA.

Immediately after irradiation, the animals were killed by decapitation after CO2 asphyxiation. Blood lymphocytes and internal organs were collected and analysed for DNA damage according to the method of Sasaki et al. [51]. Briefly, brain, liver and lungs were minced in 4.0 mL chilled homogenization buffer (pH 7.5) and homogenized at 400 rpm on ice by use of a Potter Elvehjem-type homogenizer (B. Braun, Melsungen, Germany). Subsequently, the homogenates were centrifuged (800 × g, 10 min, 4 °C). Colon cells were isolated from the mucosa by scratching, kept on ice in 2.0 mL homogenisation buffer and were used immediately for the SCGE analyses. The isolation of the lymphocytes was carried out by centrifugation as described in the human trials.

The nuclei from the different organs were analysed either under standard SCGE conditions or were treated with FPG or ENDO III (details see above). From each organ and also by the lymphocytes, three slides were prepared for experimental point and from each, 50 cells were evaluated.

2.12. Hepatic foci experiments

The animals were divided into three groups (n = 10/group) namely (i) a control group (non-irradiated which received normal water), (ii) an irradiated group (which received normal water), (iii) an irradiated group which received GA-supplemented drinking water (2.31 mg/L).

Whole body irradiation with a total dose of 12 Gy was conducted in a 60Co-source. The dose was chosen on the basis of a pilot experiment which indicated that lower doses (i.e. 6, 8 and 10 Gy) cause foci yields which are not sufficiently high to enable the detection of chemoprotective effects unless a relatively high number of animals is used (data not shown). The rats were exposed to 3 Gy for 60 s once a week over four consecutive weeks. 21 weeks after the last irradiation, the rats were killed by decapitation under CO2 asphyxiation.

Subsequently, the livers were removed and small specimens fixed in freshly prepared Carnoy’s solution (ethanol, chloroform, acetic acid; 6:3:1). After 24 h, the fixative was removed and the samples were kept for 24 h in 100% isopropanol, thereafter, the samples were embedded in paraffin. Liver sections (2.0 μm) were cut with a microtome (Leitz, Stuttgart, Germany) and stained immunohistochemically as described by Grasl-Kraupp et al. [52]. GST-p+ foci (<5 cells) were identified by anti-placental GST-stain (anti-Yp: 1:5000) under a light microscope (Nikon, Tokyo, Japan) with 400-fold magnification. The foci numbers were calculated per cm² of liver tissue and at least 1.0 cm² per animal was evaluated. From each animal two different sections from two lobes (lobus dexter and lobus sinister) were evaluated. The total area of the evaluated liver tissue was measured with an image analyser (Lucia 4.0; Nikon, Tokyo, Japan).

Before the main experiment, the optimal radiation dose was determined in order to obtain a sufficiently high number of foci.

2.13. Statistical analyses

To determine statistical differences before (T0), after (T1) GA intervention and of the different treatments (basal-damage, H2O2, FPG, ENDO III), the mean tail-lengths were analysed by ANOVA with subjects as random factors and before/after and condition as fixed factors. Homogeneity of variance was assessed by Levene’s test and norality by Kolmogorov-Smirnov tests (Lilliefors method). The extent of DNA migration attributable to FPG and ENDO III sensitive sites was calculated by subtraction of the corresponding enzyme buffer values which were determined additionally in all experiments in each participant and in each animal.

The results of SOD and GPX measurements are presented as means ± S.D. Statistical differences were assessed by paired Student’s t-test. Due to deviations from normality, GST activities (overall activity, −α and −β) were compared with the Wilcoxon’s matched-pairs tests.

We analysed also gender effects, but since they were far from being statistically significant, the factor was not included in the statistical model.

Mean tail-lengths from animal experiments were analysed by ANOVA with GA/control as one factor and enzyme treatment (FPG, ENDO III) as a second factor. Comparisons of GA vs. controls (enzyme reaction buffer alone) in experiments with the two DNA endonucleases were done by linear contrasts.

Data for the numbers and areas of hepatic foci were compared using ANOVA and linear contrasts after homogenizing the variance using logarithmic transformation followed by Dunnetts test.

Statistical analyses of other parameters (MDA, TAC, intracellular ROS, urinary isoprostane) were performed by use of the paired Student’s t-test. For all comparisons, P-values < 0.05 were considered significant. Statistical analyses were performed using Graphpad Prism 4.0 (Graphpad Software, San Diego, CA).

3. Results

3.1. In vitro SCGE

The result obtained with lymphocytes in a representative experiment under in vitro conditions is depicted in Fig. 1. Addition of GA to the cultures in the range between 0.015 and 0.15 μg/mL caused no induction of comet formation. After exposure to H2O2, a significant increase over the background was seen. When GA was added to the cultures before treatment with hydrogen peroxide, a dose dependent protective effect was observed. At the highest dose tested, the extent of DNA migration was reduced by 50%.

3.2. SCGE with human lymphocytes collected before and after consumption of GA

The results of the human trial are summarized in Table 1. The DNA migration values in the placebo group and in the GA group before the start of the intervention varied over a broad range. Although attempts were made to use an uniform group and to avoid the intake of antioxidant rich foods it can be not excluded that factors such as alcohol intake and physical exercise may have had an impact. It is notable that also in earlier trials strong inter-individual variations were seen [53].

It can be seen, that the levels of DNA migration in the placebo group were not significantly altered. In the GA group, no differences were seen when the experiments were carried out under standard conditions but the extent of DNA migration was decreased in the GA-group after treatment of the cells with H2O2 (P = 0.001) and also after treatment of the nuclei with the lesion specific enzymes FPG (P = 0.001) and ENDO III (P = 0.0002). No significant differences were seen between male and female participants.

In all experimental series protective effects were seen in most participants enrolled in the trial. Fig. 2 shows as an example the reduction of DNA migration attributable to formation of oxidized pyrimidines.

3.3. Determination of GA in plasma

GA was detectable in plasma up to 2 h after consumption. The concentration was on average 0.12 ± 2 μg/mL after 2 h, subsequently, the levels declined below the detection limit.

3.4. Enzyme measurements in plasma

The results of the enzyme measurements are summarised in Table 2. The activities of GPX and SOD increased after GA consumption by 22% (P = 0.049) and by 62% (P = 0.001) respectively, also the activity of GST-π was elevated by 61% at the end of the intervention (P = 0.028). On the contrary, the overall GST (CDNB) activity and also the activity of GST-α were not significantly enhanced.

3.5. Impact of GA consumption on different biochemical parameters

The impact of GA on intracellular ROS levels can be seen in Fig. 3. At the end of the intervention a significant reduction was observed when the cells were cultivated in presence of autologous plasma. All other biochemical parameters were not altered after intake of the phenolic, MDA levels were 1.3 ± 0.8 before and 1.2 ± 0.6 μmol/L.
Table 1
Impact of gallic acid consumption on DNA migration in human peripheral lymphocytes.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Placebo group (n=8)</th>
<th>Gallic acid group (n=8)</th>
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<tr>
<td></td>
<td>Before consumption (T0)</td>
<td>After consumption (T1)</td>
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<td>Basal DNA-damage</td>
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<td>FPG-sensitive sites\textsuperscript{d}</td>
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<td>ENDO III-sensitive sites\textsuperscript{d}</td>
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<td>7.4 ± 5.6</td>
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<td>H\textsubscript{2}O\textsubscript{2}-treatment</td>
<td>23.2 ± 5.5</td>
<td>22.7 ± 5.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All values are means ± S.D. of results obtained with all participants. From each subject, three slides were prepared and 50 cells were evaluated per slide. All measurements were carried out with a computer aided image analysis system. FPG, formamidopyrimidine DNA glycosylase; ENDO III, endonuclease III.

\textsuperscript{b} Values (before–after) that were significantly different, P<0.05 (ANOVA).

\textsuperscript{c} ∆ Alteration in %.

\textsuperscript{d} Values indicate the results obtained after subtraction of the corresponding enzyme buffer values from the values obtained after treatment of the nuclei with lesion specific enzymes.

Fig. 2. Effects of GA consumption on DNA migration in lymphocytes in ENDO III modified SCGE assays. The participants (n=16, 8/group) consumed either 0.2 mg/kg/d of GA in 500 mL water (B) or an equivalent amount water only (A) over a period of three days. After lysis, nuclei were treated with ENDO III or reaction buffer. Bars indicate the differences between the tail-lengths ± S.D. from each participant (1–8) measured after treatment of the nuclei with the lesion specific restriction enzyme ENDO III and the enzyme buffer values which were determined in each individual in parallel. From each sample three slides with enzyme treated nuclei and three slides with enzyme buffer treated nuclei were made and 50 cells were randomly analysed for comet formation per slide. Open bars = DNA migration before consumption, filled bars = DNA migration after consumption. Stars indicate statistical significance (P<0.05, ANOVA).

Table 2
Effects of supplementation of drinking water with GA on the activities of different antioxidant enzymes and on GSTs in human plasma.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Placebo group (n=8)</th>
<th>Gallic acid group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before consumption (T0)</td>
<td>After consumption (T1)</td>
</tr>
<tr>
<td>SOD U/L</td>
<td>37.9 ± 6.6</td>
<td>45.3 ± 8.9</td>
</tr>
<tr>
<td>GPx U/L</td>
<td>124.2 ± 41.2</td>
<td>126.1 ± 27.2</td>
</tr>
<tr>
<td>Total GST U/L</td>
<td>82.3 ± 13.4</td>
<td>64.6 ± 21.4</td>
</tr>
<tr>
<td>GST-α ng/mL</td>
<td>2.1 ± 1.2</td>
<td>2.2 ± 1.2</td>
</tr>
<tr>
<td>GST-β ng/mL</td>
<td>23.4 ± 1.6</td>
<td>25.4 ± 1.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All values represented means ± S.D. SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, glutathione-S-transferase. All measurements were carried out with plasma and were measured in triplicate.

\textsuperscript{b} Values that were significantly different, P<0.05 (Student’s t-test, Wilcoxon’s matched-pairs test).

\textsuperscript{c} ∆ Alteration of the enzyme activity in %.
after the intervention, the corresponding values for the TAC are 752.0 ± 76.0 and 755.0 ± 67.0 μmol/L; urinary isoprostanes were 3.49 ± 1.42 at the start of the study and 4.48 ± 2.3 ng/mg creatinine at the end.

3.6. Determination of DNA migration in blood cells and in different organs of rats

Prior to the radiation experiments we isolated lymphocytes from untreated and GA treated rats. The cells were exposed to \( \text{H}_2\text{O}_2 \) under identical conditions as the human derived cells in the intervention trial also the treatment period and the amount of GA given in the drinking water were identical. As in the human study, we found also in the peripheral blood cells of the animals significant protective effects after induction of DNA migration with the peroxide. In untreated cells the average tail length was 5.0 ± 1.9 μm, after ROS treatment the level increased to 32.0 ± 2.7 μm in cells from control animals while in GA treated animals the extent of comet formation was only 23.0 ± 1.7 μm (values are means ± S.D. of results obtained with three animals per experimental point).

The results obtained with non-irradiated and irradiated animals are summarized in Figs. 4 and 5A–D. It can be seen, that the extent of DNA migration was significantly increased when the nuclei were treated with the endonucleases. Exposure of the animals in the \(^{60}\text{Co}-\)source caused in all organs an increase of the extent of DNA migration under standard conditions compared to that seen in untreated animals, also in the experiments with lesion specific enzymes an increase of the size of the comet was seen.

Supplementation with GA did not affect basal DNA-damage in non-irradiated animals but caused a clear reduction of DNA damage attributable to oxidized purines and pyrimidines (FPG and ENDO III sensitive sites, Fig. 4A–D). The strongest inhibition of FPG sensitive-sites was seen in colons and lymphocytes (reduction by 53%, \( P = 0.003 \) and by 45%, \( P = 0.001 \)) followed by lungs and livers (reduction by 42%, \( P = 0.025 \) and by 30%, \( P = 0.044 \)).

After radiation, the protective effects of GA were more pronounced in all organs (Fig. 5A–D). In colons and lymphocytes a reduction by 55% (\( P = 0.002 \)) and by 75% (\( P = 0.001 \)) was observed under standard.

We also investigated if GA causes protection in the nervous system and measured DNA migration in nuclei isolated from brains of irradiated animals. The mean tail-lengths (TL) in the group which received drinking water was 20 ± 2.9 μm; in the GA supplemented group, DNA migration was reduced by 40% (TL 12 ± 1.2 μm, \( P < 0.001 \)).

3.7. Hepatic foci experiments

Table 3 shows the impact of GA on radiation-induced formation of GST-\( \text{p}^* \) foci. Supplementation of the drinking water with GA reduced the number of foci/cm\(^2\) by 43% (\( P < 0.001 \)) also the total foci area was significantly decreased (i.e. by 65%, \( P < 0.001 \)).

4. Discussion

Taken together, our results show that consumption of GA is associated with reduction of oxidatively damaged DNA in humans. Furthermore, the findings of the animal experiments indicate, that the phenolic prevents formation of oxidatively damaged DNA in internal organs and the results of the foci experiments provide the evidence that these effects are paralleled by protection against formation of preneoplastic hepatic lesions.

4.1. Impact of GA on DNA migration in the human intervention trial

Since the first study was published in 1996 by Duthie et al. [54], more that 90 human intervention trials have been carried out in which DNA protective properties of food components were investigated with the SCGE technique (for review see [25]). Comparisons of our findings with results from earlier investigations show that other antioxidants such as lycopene [55], epigallocatechin gallate (EGCG) [56] and also the vitamins A, C and E as well as combinations of them, carotenoids and plant derived preparations containing phenolics are by far less effective [26,27,57–59]. The daily intake levels of vitamin C and E required to cause measurable effects in peripheral lymphocytes of participants of SCGE trials were approximately 30–50 times higher than the GA dose used in the present study [19,58–60]. Furthermore, it is notable, that no DNA-protective effect was observed in a study in which the participants consumed 600 g fruits and vegetables per day over a period of 24 days [61] and also in a 21-day trial with daily consumption of 330 g of a mixed fruit/vegetable juice no protection was seen [62]. These comparisons indicate that GA is more active in regard to prevention of oxidative DNA damage as other dietary factors.

4.2. Results of the animal experiments

The results of the present in vivo experiments in which the animals received GA at an identical daily dose as that consumed by the participants show that protection against oxidized purines occurs also in liver, lungs and colon. Furthermore, we observed in the radiation experiments a significant decrease of DNA migration (40%) in nuclei from the brain, indicating that GA is also protective in the nervous tissue. In this context it is notable that it has been postulated that diseases such as Alzheimer’s and Parkinson’s disease (PD) are causally related to ROS [1,63]. PD is characterised by a selective degeneration of dopaminergic neurons in certain parts of the brain. It is assumed that the oxidation of the neurotransmitter dopamine generates ROS which cause cell death of these neurons and it was shown in in vitro experiments with a human derived neuroblastoma cell line that GA prevents the cytotoxic effects caused by 6-hydroxydopamine. The authors of this study conclude that GA may prevent oxidative stress injury in neurodegenerative diseases [61].

Only a limited number of SCGE studies, for example with green tea extract and vitamin C, have been published in which inhibition of DNA damage by dietary constituents was investigated in rodents.
bars either intracellular organs. Table [64–67]. As in the case of the human studies, comparisons indicate that GA is more potent in regard to prevention of oxidative damage. The metabolic rate of rats is higher than that of humans [68]. Therefore it is not possible to translate the results of the animal experiments directly to humans. However, the main purpose of the in vivo study was to find out if the protective effects seen in the lymphocytes are paralleled by reduced DNA damage in inner organs. Our findings show that this is indeed the case and can be taken as an indication that also in humans protective effects can be expected in colon, liver and lungs.

4.3. Induction of antioxidant enzymes and reduction of intracellular ROS levels by GA

Only few investigations with rodents have been published with GA and none of them concerns its DNA protective properties. In all of them the doses exceeded those used in the present study by 2–3 orders of magnitude. Some of these investigations provide evidence for antioxidant properties of GA, for example the reduction of CCL\textsubscript{4} induced cytotoxicity in rat livers was interpreted as a consequence of prevention of LP [23] and in a model with senescence accelerated mice, prevention of age related formation of LP products and reversion of the decline of antioxidant enzymes was found [69]. Furthermore, induction of enzymes such as CAT, SOD and GPx was observed in hepatic and coronary tissue of GA treated rats [22,70].

As described above, we found also in the human intervention trial induction of GPx and SOD as well as an increase of the activity of GST–π. The latter enzyme is not only involved in the inactivation of radicals but also in the detoxification of DNA reactive carcinogens. The SOD activities in plasma reflect extracellular superoxide dismutase (EC SOD) which is synthesized in a variety of tissues including blood vessels, lungs and kidneys and to a lesser extent also in the liver [71]. It is well documented that the EC SOD plays a key role in ROS protection and cell signaling and it was postulated in a variety of studies that increased levels are beneficial in prevention and treatment of a variety of diseases (for details see [71]).

Table 3

<table>
<thead>
<tr>
<th>Treatment group(^b)</th>
<th>Number of foci/cm(^2)</th>
<th>(\Delta(%)(^c)</th>
<th>Total area (mm(^2)/cm(^2))</th>
<th>(\Delta(%)(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.47 ± 0.1</td>
<td>–</td>
<td>0.03 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>γ-irradiation</td>
<td>28.95 ± 8.7(^e)</td>
<td>–</td>
<td>18.03 ± 2.3(^f)</td>
<td>–</td>
</tr>
<tr>
<td>GA + γ-irradiation</td>
<td>16.56 ± 3.2(^g)</td>
<td>–33</td>
<td>6.23 ± 1.4(^h)</td>
<td>–65</td>
</tr>
</tbody>
</table>

\(^a\) Data are means ± S.D. of results obtained with 10 animals/group.

\(^b\) The animals received 2.31 mg/L GA in drinking water over a period of 8 days before radiation (12.0 Gy).

\(^c\) Significant difference compared to the control group

\(^d\) Significant difference between the GA-group and the group without GA supplementation after irradiation (P < 0.05, Dunnett’s test).

\(^e\) \(\Delta\) Alteration of the number of foci/cm\(^2\) in %.

\(^f\) \(\Delta\) Alteration of the total foci area in %.
in the results section the overall (CDNB) GST levels and GST-α activities were not altered after consumption of GA. Measurements with CDNB represent the sum of the activities of different isozymes and reflect primarily the situation in the liver where the highest activity is found. Also the GST-α levels are high in hepatic tissue, while GST-π which was significantly induced by GA is synthesized in other organs such as spleen, kidneys and stomach [72]. The main source of plasma GPx which was significantly increased after GA intake (i.e. by 22.2%) is the kidney. Lower amounts are also produced in other organs including liver, pancreas, brain and lungs [73].

The observation of simultaneous induction of the different enzymes in plasma does not allow to draw firm conclusions which of them accounts for the reduction of DNA damage which we found in the peripheral blood cells and in the internal organs. However, they indicate that enzymatic inactivation of ROS accounts for the DNA protective effects.

4.4. Mechanistic aspects

The impact of GA on oxidative damage of macromolecules and its acute toxic effects have been investigated in a number of in vitro studies. It was repeatedly found in experiments with high doses that the GA induces apoptosis in human derived cell lines [16,74–78], furthermore, also induction of DNA damage was also reported [19,20]. These effects were explained by formation of ROS and reduced by addition of scavengers and antioxidant enzymes [16,74–76]. In contrast to these investigations several studies were published which showed that physiologically more relevant concentrations (≤0.1 μM) are DNA protective and prevent apoptosis and it was postulated that these effects are due to scavenging of radicals and/or induction of DNA repair enzymes [19,21,79]. In the experiments concerning the prevention of programmed cell death, the cells were damaged with a ROS generating neurotoxin after pretreatment with GA and the authors conclude that the phenolic prevents formation of damaged cells due to its antioxidant properties [79].

The results of the in vitro experiments (Fig. 1) show that GA protects human lymphocytes against ROS induced DNA damage. The short exposure period which was used in these experiments is not sufficient to induce antioxidant enzymes and protective effects are in this case probably due to direct scavenging. However, the lack of alterations of the TAC in plasma and of the LP markers (MDA in plasma and urinary 8-epi-PGF_2α,1) after consumption of GA and the observation of changes of intracellular parameters such as reduction of formation of endogenously formed oxidised DNA bases in the intervention trial and in the animal experiments indicate that indirect mechanisms account for the in vivo effects. Also the observation of a decrease of intracellular ROS levels in presence of autologous plasma (which is probably due to increased activities of antioxidant enzymes) as well as the increase of the activities of SOD, GPx and GST-π suggests that the antioxidant defence system is induced by GA. In this context it is notable that it was shown in rats that the phenolic induces the transcription Nrf2 [22] which controls genes enrolled in the protection against ROS.
As mentioned in the results section, we found a substantially higher level of intracellular ROS when the cells were cultivated in absence of plasma. This can be explained by inactivation of radicals by plasma components such as proteins and the same observation has made in earlier studies [80,81]. The reason why we found reduction after GA intake only when the cells were grown in presence of plasma can be explained by increased activities of antioxidant enzymes (see above).

In the present study and also in several earlier intervention trials with different dietary components significant reduction of oxidative DNA damage and/or prevention of ROS induced comet formation was detected while no reduction of DNA migration was seen under standard conditions in the same experiments probably due to the fact that oxidised DNA is rapidly repaired in healthy individuals (for review see [25]). However, the results of the radiation experiments with the rats (Fig. 5) indicate that GA is also effective when DNA damage (induced partly by ROS) is severe enough to cause comet formation under standard conditions (which reflect single and double strand breaks as well as apurinic sites [33].

Dotan et al. [82] compared the effects of studies in which different parameters of the redox status were measured in parallel and found in general only poor overlaps. For example, alterations of parameters of LP and of endpoints of oxidatively damaged DNA did not correlate and also parameters that reflect the antioxidant capacity of plasma were altered independently. The reasons for these discrepancies are differences in mode of action of different antioxidants (direct vs. indirect) in the oxidation of different macromolecules and also of the distribution of antioxidants and oxidants in the body and in cellular compartments.

4.5. Prevention of preneoplastic lesions in the liver by GA

As shown in Table 3, we observed reduction of formation of GST-p+ foci with a low amount of GA (the daily dose was identical to those given in the SCGE studies with humans and rats). Since the carcinogenic effect of ionising radiation is partly due to intracellular formation of ROS, our findings can be taken as an indication that the prevention of formation of the preneoplastic lesions in the liver is due to protection against oxidatively damaged DNA. Only one study has been published so far which indicates indirectly that GA has anticarcinogenic properties. Jagan and co-workers [83] reported that GA treatment (10 mg/kg b.w.) of rats leads to reduction of diethylnitrosamine-induced cell proliferation. Since superoxide plays a role in the induction of cell division and DNA damage by this nitrosamine [84], it is conceivable that the antioxidant properties of GA involve detoxification of this radical.

It is assumed that ROS are involved in both, initiation as well as tumour promotion [85], therefore the results of the foci experiments were not unexpected. However they are interesting as they indicate that the prevention of oxidative DNA by GA is paralleled by protection against malignant transformation.

4.6. Possible impact of GA on human health

Taken together, our findings suggest that GA may protect against cancer and other ROS associated diseases. In this context it is notable that the phenolic was postulated to be the active component of herbal remedies used in traditional medicine such as *Terminalia cebula* [24] and *Terminalia belerica* [23], rose flowers [69] and *Toona sinensis* [16]. Furthermore, the prevention of DNA damage seen with sumach (*Rhus coriaria*), a common spice which is also used in folk medicine the Middle East [5] and with carob fibre from *Ceratonia siliqua* were attributed to their GA contents [9].

It has been estimated that the daily intake of GA in Southern Germany is 18 mg/P/d [86]. Since the dose we used in the present experiments was even lower, our findings suggest that uptake of the phenolic via food consumption contributes substantially to the maintenance of the redox status of GA and may improve the health status of individuals under oxidative stress. Since GA is contained in a number of foods, it is easily possible to increase its daily intake by dietary measures. In order to further substantiate the findings of the present study in humans, a larger intervention trial is planned in which also further attempts will be made to elucidate the molecular mechanisms in more detail (e.g. alterations of DNA repair processes) and the time course of the effects.

Conflict of interest statement

There are no conflicts of interest to report.

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Appendix A. Supplementary data


References

