Radioprotection of DNA by Glycyrrhizic Acid Through Scavenging free Radicals

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Glycyrrhizic acid/Pulse radiolysis/Radiation protection/Plasmid DNA/DNA strand breaks/Comet assay

Gamma-radiation induced strand breaks in plasmid pBR322 DNA. Glycyrrhizic acid (GZA) protected plasmid DNA from radiation-induced strand breaks, as the disappearance of super-coiled (ccc) form was prevented by the compound with a dose-reduction factor of 2.04 at 2.5 mM concentration. Studies of comet assay on human peripheral blood leukocytes exposed to gamma radiation in the presence and absence of glycyrrhizic acid ex vivo revealed that this compound protected the cellular DNA from radiation-induced strand breaks in a concentration-dependent manner. An intraperitoneal administration of the GZA to mice one hour before exposure to gamma radiation protected cellular DNA from radiation-induced strand breaks in peripheral blood leukocytes and bone marrow cells, as revealed by comet assay. Pulse radiolysis studies indicated that glycyrrhizic acid offered radioprotection by scavenging free radicals. The rate constants for the reaction of glycyrrhizic acid with OH· and eaq– are 1.2 × 10¹⁰ M⁻¹ s⁻¹ and 3.9 × 10⁹ M⁻¹ s⁻¹, respectively.

INTRODUCTION

Root extracts of the plant Glycyrrhiza glabra L., known as Yashtimadhu, in Ayurveda have been used for curing various diseases because of its antiinflammatory, antibacterial, antiviral, and immune-modulating activities¹. The extract, generally called liquorices, is widely used as a sweetener in food products and chewing tobacco. The active compounds of the extract have been reported to have immuno-modulating² antioxidant³,⁴. Our earlier studies have revealed the radio protective effect of the extract on gamma radiation induced DNA and membrane damages⁵). One major component of the extract is glycyrrhizic acid (Scheme I and its absorption spectra in Fig. 1). In biological systems, most of the damage induced by gamma rays is indirect and mediated through free radicals that interact with important macromolecules, including DNA and membranes⁶,⁷. Membrane damage occurs mainly because of the peroxidation of membrane lipids. In the present study we have investigated the mechanism of radiation protection of DNA by glycyrrhizic acid (GZA) in vitro, ex vivo, and in vivo. The effect of GZA on the production of gamma radiation induced strand breaks in plasmid pBR322 DNA in vitro was monitored by agarose gel electrophoresis from the disappearance of supercoiled form (ccc) of DNA. The radiation-induced strand breaks in cellular DNA of human peripheral blood leukocytes was examined in ex vivo studies by the use of alkaline comet assay. Studies on in vivo radioprotection of DNA were undertaken in mice by an ip administration of GZA before whole-body irradiation and measuring strand breaks in the cellular DNA of peripheral blood leukocytes and bone mar-

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row cells by alkaline comet assay. To understand the molecular mechanisms of radioprotection by GZA, the bimolecular rate constants for the reactions of primary radicals of water radiolysis with GZA were determined with the pulse radiolysis technique.

**MATERIALS AND METHODS**

**Reagents**

Plasmid pBR322 DNA was purchased from Bangalore Genei, Bangalore, India. Glycyrrhizic acid, propidium iodide, and ethylenediaminetetraacetic acid were obtained from Sigma Chemical Co., USA. Other reagents were of analytical grade obtained from local manufacturers.

**Collection of human blood**

Human blood samples were collected from three healthy nonsmoking volunteers, having a mean age of 25 ± 2 years, by the finger prick method and stored in heparinized eppendorf tubes at ice temperature.

**Animals**

Male Swiss mice, 8–10 weeks old and weighing 20–25 g each, were selected from an inbred group maintained under standard conditions of temperature (25 ± 2°C) and humidity, with a 12-hour light and 12-hour dark cycle. The animals were provided with food and water ad libitum. Usually four animals were housed in each sterile polypropylene cage containing a sterile paddy as bedding. All animal experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of the Government of India on the use of animals in scientific research.

**Gamma irradiation**

Plasmid pBR322 DNA (20–25 µg/ml) in 0.1M sodium phosphate buffer pH 7.0 was exposed to 60Co-gamma rays in a Gamma Cell 220 (AECL, Canada) at a dose rate of 9.6 Gy per min in the presence and absence of GZA. For in vivo experiments, the animals after ip administration of GZA (4 mg / kg body weight in double distilled water) were kept in a well-ventilated, acrylic box and whole-body exposed to 4 Gy in a Junior Theratron unit (AECL, Canada) having a dose rate of approximately 0.5 Gy/min. Ex vivo irradiation of human peripheral leukocytes in the presence and absence of glycyrrhizic acid was also done in the Junior Theratron unit.

**Effect of glycyrrhizic acid on radiation-induced DNA damage in blood leukocytes and bone marrow cells**

The animals were divided into the following groups:
1. DDW+ sham irradiation
2. DDW+ 4 Gy irradiation
3. Glycyrrhizic acid + sham irradiation
4. Glycyrrhizic acid + 4 Gy irradiation

One hour after the administration of DDW or GZA, animals were whole-body exposed to 0 (sham-irradiation) or 4 Gy by use of the Junior Theratron unit in a well-ventilated acrylic box. After irradiation, all four groups of were kept back in a polycarbonate cage. All animals were sacrificed by cervical dislocation after two hours of irradiation. Blood was collected in heparinized eppendorf tubes by heart puncture, and bone marrow cells were collected from the femurs in ice-chilled 10 mM potassium phosphate buffer, pH 7.4.

**Analysis of DNA damage**

Radiation-induced damage to plasmid DNA was determined by electrophoresis in 1% agarose gel. The ethidium bromide stained DNA bands were analyzed by the use of Syngene software.

**Measurement of DNA damage by the use of Single-Cell Gel Electrophoresis (Comet Assay)**

The DNA strand breaks in the bone marrow cells and blood leukocytes were measured by the use of single-cell gel electrophoresis (comet assay) based on the method of Singh et al. 2000, with some modification. Fully frosted microscope slides (Gold Coin, Mumbai) were covered with 200 µl of 1% normal melting agarose (NMA) in PBS at 45°C, immediately cover –slipped and kept at 4±2°C for 10 min to allow the agarose to solidify. The removal of the cover slip from the agarose layer was followed by an addition of a second layer of 200 µl of 0.5% low-melting agarose (LMA) containing approximately 10² cells at 37°C. Cover slips were placed immediately, and the slides were placed at 4°C. After a solidification of the LMA, the cover slips were removed and the slides were placed in the chilled lysing solution consisting of 2.5 M NaCl, 100 mM Na₂-EDTA, 10 mM Tris-HCl, pH 10, 1% DMSO, 1% Triton-X100, and 1% Triton X-100.
sodium sarcosinate for 1 hour at 4°C. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na₂-EDTA, and 0.2% DMSO, pH ≥13.0). The slides were equilibrated in the same buffer for 20 min, and electrophoresis was carried out at 4 V, 350 mA for 15 min. After electrophoresis, the slides were washed gently with 0.4 M Tris-HCl buffer, pH 7.4, to remove the alkali. They were stained with propidium iodide and visualized with a fluorescent microscope (Carl Zeiss Axioskop) with bright field phase contrast and an epifluorescence facility attached to a high-performance JVG TK 1280E camera. The integral frame grabber used in this system (Cvfb01p) is a PC-based card, and it accepts color composite video output of the camera. Fifty cells/slide were captured. The quantification of the DNA strand breaks of the stored images was done with CASP software, by which a percent of DNA in the tail, tail migration, tail moment, and Olive tail moment could be obtained directly.

Pulse Radiolysis studies

Pulse radiolysis studies were carried out by irradiating solutions in rectangular quartz cells. The pulse radiolysis setup consists of an electron linear accelerator (Viritech Ltd., U.K.) capable of giving single shots of 50 or 500 ns or 2 μs of 7 MeV electron pulses. The pulse irradiates the sample contained in a 1 cm × 1 cm suprasil quartz cuvette kept about 12 cm from the electron beam window, where the beam diameter is approximately 1 cm. The transient changes in the absorbance of the solution caused by the electron pulse are monitored with the help of a collimated light beam from a 450 W xenon arc lamp. The output from the PMT is fed through a DC offset circuit to the Y input of an L & T storage scope that can transfer 400 mega-samples/sec on each input channel at a 250 ns/div time base range with a sensitivity of 2mV/div and having a bandwidth of 100 MHz. However, further details of the LINAC can be seen elsewhere.

An aerated 0.05 mol dm⁻³ KSCN solution was used for dosimetry, and the (SCN)₂⁻ radical was monitored at 500 nm. We calculated the absorbed dose per pulse, assuming G.e. for the (SCN)₂⁻ radical to be 23889 dm³ mol⁻¹ cm⁻¹ per 100 eV, where G is the radiation chemical yield expressed as the number of molecules formed or destroyed per 100 eV of energy absorbed and ε is the molar absorptivity. The dose employed in the present study, unless otherwise stated, was a typical 16 Gy per pulse.

On irradiation of H₂O, the following primary radicals are produced

\[
H_2O \rightarrow e_{aq}^-, H^+, OH^- \tag{1}
\]

For studying the reaction of e_{aq}^- with glycyrrhizic acid, we used an N₂-bubbled aqueous solution containing 0.1 mol dm⁻³ 2-propanol. OH⁻ and H⁺ radicals get scavenged by 2-propanol. We measured the rate of reaction of e_{aq}^- with glycyrrhizic acid by monitoring the decay of e_{aq}^- at 700 nm.

To selectively produce OH⁻ radicals, we saturated solutions with N₂O before pulse irradiation. This resulted in a scavenging of e_{aq}^-, and under these conditions the yield of OH⁻ radicals is 90% of the total radical yield; G(OH⁻) = 5.4 mol/100 eV = 5.6 × 10⁻⁷ mol J⁻¹. The remaining 10% contribution is of H⁺ atoms G(H⁺) = 0.6 mol/100 eV = 0.62 x 10⁻⁷ mol J⁻¹. We measured the rate of reaction of OH radicals with glycyrrhizic acid by monitoring the buildup kinetics at 320 nm.

Fig. 2. Agarose gel electrophoresis pattern of pBR322 DNA exposed to various doses of γ-radiation in the presence and absence of 2.5 mM GZA (negative of the photograph). The upper and lower bands depict the open circular (oc) and the supercoiled (ccc) forms, respectively. Lanes 1, 3, 5, 7, 9, and 11 show the results of pBR322 degradation to oc form at the radiation doses of 10, 20, 30, 40, 50, and 100 Gy, respectively, without the presence of the compound. Lanes 2, 4, 6, 8, 10, and 11 show the effect of the presence of GZA at the same radiation doses, respectively. Lane 13 is the control lane (without radiation).

Fig. 3. Presents the quantified data from Fig. 1 analyzed by Syn gene Software (Syngene Inc., USA). Each point is the average of the three independent experiments. The dose-reduction factor for glycyrrhizic acid (2.5 mM) for the protection of the DNA in vitro was determined to be 2.04.
The rates of reactions were determined by carrying out the experiments with at least three different concentrations of glycyrrhizic acid, varying by at least a factor of 4. Bimolecular rate constants were derived from plots of the first-order rates vs. concentration. The calculated bimolecular rates are within the limits of ± 15% experimental error.

Statistical analysis

The results are represented as mean ± standard error of mean (mean ± SEM). ANOVA was done by using origin 5.0. The results are significant at p<0.01 and p<0.05

RESULTS

The reverse of a photo negative of the agarose gel electrophoresis of pBR322 DNA exposed to various doses of γ-radiation in the presence and absence of 2.5 mM GZA is given in Fig. 2. As compared to control, the exposure of plasmid DNA to gamma radiation decreased the relative intensity of the ccc form of DNA. This decrease was dependent on the increase in the radiation dose, as could be seen in lanes 1, 3, 5, 7, 9, and 11. The presence of GZA along with DNA during irradiation prevented this decrease of the ccc form, as evident in lanes 2, 4, 6, 8, 10, and 12. This would suggest that GZA offered protection to DNA in vitro against gamma radiation induced strand breaks. The reduction in the quantity of the supercoiled (ccc) form of plasmid DNA is directly related to the radiation-induced damage, particularly strand breaks in DNA. There was a dose-dependent reduction of the ccc form of plasmid DNA when exposed to 0-100 Gy of gamma radiation, and the presence of 2.5mM GZA prevented this loss of ccc form, as can be seen in Fig. 3. The dose-reduction factor (DRF) for the DNA at 2.5mM GZA calculated from the data is 2.04.

An exposure to γ-radiation ex vivo induces damage to the DNA of human peripheral blood leukocytes, as can be

![Graphs showing DNA damage](image_url)

Fig. 4. Effect of Various concentrations on γ-radiation-induced DNA damage in human blood lymphocytes, estimated by comet assay in terms of the percent DNA in tail (A), tail length (B), tail moment (C), and Olive tail moment (D). 1: control; 2: GZA (1.0 mM) alone; 3: 2 Gy radiation alone; 4, 5, 6, 7, 8, and 9: 2 Gy radiation + 10, 7.5, 5, 2.5, 1.0, and 0.5 mM GZA, respectively.
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inferred from the data on comet assay presented in Fig. 4. An exposure of human peripheral blood leucocytes to 2 Gy γ-radiation ex vivo resulted in increases of the comet parameters, such as % DNA in tail, tail length, tail moment, and Olive tail moment, and the presence of GZA during irradiation inhibited these increases in a concentration-dependent manner (Fig. 4, a–d). These results thus suggest a protection of cellular DNA by GZA from radiation damage.

Figures 5 and 6 depict the results of comet assay performed on blood leukocytes and bone marrow cells from whole-body irradiated mice treated with GZA. The whole-body exposure of animals to gamma radiation (4 Gy) resulted in an increase in the comet parameters (such as % DNA in tail, tail length, tail moment, and Olive tail moment) of the cells of blood leucocytes and bone marrow as a result of damage to cellular DNA. When GZA was administered 1 hr before irradiation, there was a significant decrease in comet parameters in blood leucocytes and bone marrow cells of irradiated mice (Figs. 5 and 6). When the animals were exposed to gamma radiation (4 Gy), % DNA in the tail was increased from 3.117 ± 0.45 to 8.133 ± 0.54, tail length was increased from 8.631 ± 0.94 to 26.158 ± 1.49, tail moment was increased from 0.689 ± 0.21 to 3.475 ± 0.44, and Olive tail moment was increased from 0.869 ± 0.15 to 3.626 ± 0.32 in blood cells. But the administration of GZA 1 hour before irradiation brought these parameters down to levels of 6.496 ± 0.54, 22.016 ± 1.39, 2.728 ± 0.41, and 2.555 ± 0.25, respectively, in the irradiated animals (Fig. 5, A–D).

In the bone marrow cells of animals exposed to radiation (4 Gy), % DNA in tail, tail length, tail moment, and Olive

![Fig. 5. Effect of Glycyrrhizic acid on DNA damage in murine blood leucocytes assayed by Comet assay: (a) % DNA in tail; (b) Tail length; (c) Tail moment; (d) Olive tail moment. (Why small letters here (a), (b), etc., and capital letters in Figs. 4 and 6 (A), (B), etc.? (And why capitalize Tail length and Tail moment here and below when you didn’t above in Fig. 5? Please be consistent.)](image-url)
The tail moment was increased from the respective control value 2.265 ± 0.4, 9.971 ± 1.12, 0.787 ± 0.27, and 0.832 ± 0.18 to 5.412 ± 0.29, 20.19 ± 0.95, 1.704 ± 0.16, and 2.001 ± 0.12. The administration of GZA 1 hour before irradiation brought these levels down to 4.702 ± 0.32, 18.376 ± 0.088, 1.392 ± 0.16, and 1.73 ± 0.13, respectively, in the irradiated animals (Fig. 6, A–D).

The mechanism of radioprotection by GZA was investigated by pulse radiolysis studies. The transient absorption spectra obtained on the reaction of glycyrrhizic acid with OH radicals is presented in Fig. 7. The bimolecular rate constants for the reactions of OH• and eaq− with GZA were determined as explained in materials and methods and found to be $1.2 \times 10^{10}$ M$^{-1}$ s$^{-1}$ and $3.9 \times 10^9$ M$^{-1}$ s$^{-1}$, respectively. The transient produced on reaction with OH radical shows an absorption maximum at 320 nm and did not decay up to 2 milliseconds, both in the presence and absence of air. However, we could observe no transient formation on the reaction of GZA with eaq− in the wavelength region of 280 nm to 700 nm under our experimental conditions.

To see the radioprotection of GZA, pulse radiolysis experiments were carried out. Experimental conditions were chosen in such a way that the electron and the OH radicals reacted selectively with Thymine in the presence of GZA. It was observed that eaq− and OH• radical adducts of thymine were found to be nonreactive toward glycyrrhizic acid (our unpublished observations). The results thus indicate that glycyrrhizic acid offers radioprotection by a scavenging of free radicals.
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DISCUSSION

DNA constitutes the primary vital target for cellular inactivation of living systems by ionizing radiation. The present study shows that DNA is protected from the deleterious effects of \( \gamma \)-radiation by GZA in the in vitro, ex vivo, and in vivo conditions of radiation exposure. Ionizing radiation-induced damages to cellular DNA are mainly strand breaks of the double- and single-strand types, base damages, elimination of bases, and sugar damage. In the in vitro studies, it was found that when the plasmid pBR322 was exposed to \( \gamma \)-radiation, the ccc form of the molecule was converted to the oc form, with a difference in the mobility in the agarose gel because of the induction of strand breaks in the DNA. Several phytochemicals have been shown to be radioprotectors.

Nontoxic compounds that can protect DNA against ionizing radiation have considerable potential as radioprotectors and could be of use in preventing diseases like cancer and degenerative diseases arising from gene mutations. GZA does not induce DNA damage by itself, but it inhibits the induction of single-strand breaks in DNA by \( \gamma \)-radiation. The ability of GZA to protect DNA in vitro is determined, as a dose-reduction factor (DRF), from the dose response curve for undamaged ccc DNA remain after exposure to various doses of radiation. The DRF for the plasmid DNA at 2.5 mM GZA is 2.04.

The damages by ionizing radiation to DNA can cause the loss of viability of the cells exposed to radiation. The alkaline comet assay is an elegant and effective technique to monitor the extent of the DNA damage and its protection. When the human leucocytes are exposed to \( \gamma \)-radiation ex vivo, the cellular DNA undergoes damage, as reflected in the increase of the comet parameters (tail length, tail moment, % DNA in the tail, among others). GZA’s presence during irradiation of the cells decreases the comet parameters in a concentration-dependent manner indicative of radiation protection.

Whole-body exposure of animals to gamma radiation (4 Gy) resulted in an increase in the comet parameters of cells of various tissues and tumors because of damage to cellular DNA. The IP administration of GZA to mice before whole-body irradiation protected the mice because there was a decrease in the comet parameters in various tissues, such as bone marrow and blood cells. The results presented in our work clearly show that the GZA protects bone marrow cells from the radiation-induced damages. This would suggest that GZA could be a potential drug for the protection of the hemopoietic system from radiation-induced lesions.

To understand the mechanism of radioprotection by GZA at a molecular level, we have studied by pulse radiolysis technique the transient absorption spectra of glycyrrhizic acid on reaction with OH radicals. Glycyrrhizic acid reacted with OH\(^-\) and \( \text{e}_{aq}^-\) with rate constants \( 1.2 \times 10^{10} \text{ M}^{-1} \text{s}^{-1} \) and \( 3.9 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \), respectively. The results thus indicate that glycyrrhizic acid offers radioprotection by the scavenging of free radicals.

GZA is the main active ingredient of liquorice, a natural sweet substance originating from the root of the genus *Glycyrrhiza*. This genus contains about 14 species with varying degrees of sweetness, such as *gabra* (Spanish), *glandulifera* (Russian and Persian), *echinata* (German), *uralensis* (Asian), and *lepidota* (American). Among them, the main source of medicinal liquorice is *Glycyrrhiza glabra*. Liquorice has been used since ancient times as an herbal remedy for coughs, peptic ulcers, constipation, stomachache, arthritis, insomnia, depression, fatigue, asthma, and liver problems. It is administered in such forms as liquid extract, powder, concentrate, infusion, tablet, capsules, tincture, candy, gum, tea, soft drinks, and alcoholic drinks. The undesirable side effects of liquorice include water and sodium retention, hypertension, hypokalemia, and alkalosis, and these are reversible if it is discontinued. Liquorice contains 0.2% GZA. An acceptable daily intake of GZA for human subjects was estimated to be 0.2 mg/kg body weight. Our findings reveal that GZA protects cellular DNA against radiation-induced damage. One deleterious consequence of DNA damage from exposure to ionizing radiation is the induction of cancer. Protecting cellular DNA from radiation damage might result in the prevention of the cancers induced by the radiation. Recently it has been reported that diagnostic X-rays -though they provide great benefits -do cause a risk of cancer. GZA or liquorice administration to the patients undergoing medical X-ray exposures might reduce the incidence of these cancers.
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