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ORIGINAL RESEARCH

In vitro studies on protective effect of *Glycyrrhiza glabra* root extracts against cadmium-induced genetic and oxidative damage in human lymphocytes

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Abstract Cadmium is a modern environmental contaminant that is toxic and carcinogenic. *Glycyrrhiza glabra* is a traditional medicinal herb which grows in the various parts of the World. Recent studies demonstrated that *G. glabra* has antifungal, antimicrobial, antioxidant, and powerful antiinflammatory features. The purpose of this study was to investigate the genetic safety of extracts from *G. glabra* and its effects on cadmium (as $CdCl_2$) induced genotoxicity. Therefore we evaluated the capability of *G. glabra* extract to inhibit the rate of micronucleus (MN), sister chromatid exchange (SCE) formations induced by $CdCl_2$. Moreover, to assess the effects of *G. glabra* on cell viability and oxidative status, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and total antioxidant capacity (TAC) assays. Our results showed that there were significant increases ($P < 0.05$) in both SCE and MN frequencies of cultures treated with $CdCl_2$ (5 ppm) as compared to controls. However, co-application of *G. glabra* extract (5, 10 and 20 ppm) and $CdCl_2$ resulted in decreases of MN and SCE rates as compared to the group treated with $CdCl_2$ alone. Again, the results of MTT and TAC assays clearly indicated dose dependent ameliorative effects of *G. glabra* extracts against $CdCl_2$ toxicity. In conclusion, this study demonstrated for the first time that *G. glabra* extracts provided increased resistance of DNA against $CdCl_2$ induced genetic and oxidative damage in human lymphocytes. So, the risk on target tissues of $CdCl_2$ could be reduced and ensured early recovery from its toxicity.

Keywords Cadmium · *Glycyrrhiza glabra* · Genotoxicity · Human lymphocytes · Oxidative stress · Protective effect

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Introduction

Glycyrrhiza glabra L. formerly known as licorice or sweetwood, native to the Mediterranean and certain areas of Asia, is a tall shrub of the Leguminosae family (Fenwick et al. 1990; Olukoga and Donaldson 1998). Roots of *G. glabra* have demulcent, antacid, anti-ulcer (Nadkarni 1998), anti-inflammatory, expectorant, tonic, diuretic, laxative, and sedative properties (Hikino 1985). They also possess antipyretic (Lata et al. 1999), antimicrobial, antiherpes (Ceremelli et al. 1996), and anxiolytic (Ambawade et al. 2001) activities. Glycyrrhizin, a triterpene saponin, possesses antiviral activity

(Hirabayashi et al. 1991). Licorice (*G. glabra*) is one of the most important crude drugs in the world, and its major triterpene saponin, glycyrrhizin, is a well-known natural sweetener and pharmaceutical (Gibson 1978; Shibata 2000). In addition, extracted licorice, containing glycyrrhizin, is used as an additive for flavoring and sweetening tobacco, candies, chewing gum, toothpaste, and beverages all around the world (Baltina et al. 2003; Rauchensteiner et al. 2005). *G. glabra* and *G. uralensis* are known to be the major glycyrrhizin-producing species (Shibata 2000). The roots of licorice are rich sources of flavonoids, in particular, prenylated flavonoids, such as glabridin and glabrene. This plant has been long used to treat fevers, liver ailments, dyspepsia, constipation, gastric ulcers, sore throats, spasm, asthma, bronchitis, Addison's disease and rheumatoid arthritis. Moreover, *G. glabra* is widely used in Indian Medicine (Schulz et al. 1998; Wang et al. 2000; Anon 2005).

Heavy metals are chemical elements that are commonly found in our environment, which are poisonous at high doses and even at fairly low concentrations cause human health problems. Cadmium (Cd) is one such highly toxic heavy metal known to have impact on human and animals (Loser 1980; Watanabe et al. 1986). Cd is a particularly dangerous pollutant due to its high toxicity and great solubility in water that can cause emphysema, anemia, osteoporosis, chronic rhinitis (Duxbury 1985; Jiang et al. 2001; Waisberg et al. 2003). According to the International Agency for Research on Cancer Cd is suspected as co-mutagen and carcinogen in human (IARC 1993). Moreover, exposures to high Cd concentration have been found to be carcinogenic, mutagenic and teratogenic for a large number of animal species (Degraeve 1981). Numerous experimental studies have shown the genotoxicity of Cd salts (Fojtova and Kovarik 2000; Seoane and Dulout 2001). Several studies, indicated that Cd damaged the nucleolar structure, DNA and RNA in both animal and plant cells (Misra et al. 1998; Jonak et al. 2004). Although Cd is a systemic poison known to affect many cell functions (Berti and Averbeck 2006), the cellular and molecular effects of a prolonged exposure to this metal are not completely understood.

Recently, extensive efforts were made to investigate therapeutic substances capable of reducing the genotoxicity of various natural and man-made mutagens in human life (Turkez et al. 2005; Turkez and Geyikoglu 2010b). Also, these include antibody, fatty acids, minerals and vitamins (Edenharder et al. 1999; Rao et al. 2001; Yoshida et al. 2010; Turkez et al. 2012a, b, c, d). Concomitant treatment with antioxidants provided protection against oxidative damage by mutagens in experimental animals (Abubakar et al. 2003; Esparza et al. 2003; Geyikoglu et al. 2005; Turkez and Geyikoglu 2010a). So far, antioxidants have attracted much interest with respect to their protective effect against free radical damage that may be the cause of many diseases including cancer (Shon et al. 2004). Since the complete avoidance of exposure to $CdCl_2$ is very difficult, chemoprevention is an attractive strategy for protecting humans and animals from the risk of cancer caused by exposure to this heavy metal. Besides, many efforts are being made to investigate therapeutic substances capable of reducing the toxicity of man-made or natural mutagens in human life. Thus, this study investigated the efficacy of *G. glabra* extract against $CdCl_2$-induced DNA and oxidative damages.

Materials and methods

Experimental design

Blood samples were obtained by veinpuncture from five healthy non-smoking donors. Human peripheral blood lymphocyte cultures were set up according to the protocol with slight modifications described by Evans and O'Riordan (1975). The heparinized blood (0.5 ml) was cultured in 6 ml culture medium (Chromosome Medium B, Biochrom®, Berlin, Germany) with 5 µg/l of phytohemagglutinin (Biochrom®). $CdCl_2$ was purchased from Sigma (St. Louis, MO, USA; CAS No. 7790-78-5). *G. glabra* was purchased in ready package from local markets. For water extraction of *G. glabra*, 20 g roots was mixed with 400 ml distilled and boiling water using magnetic stirrer for 15 min. Then extract was filtered over Whatman No. 1 paper. Then, $CdCl_2$ (5 ppm) and *G. glabra* extracts (5, 10 and 20 ppm) were added into culture tubes separately and together. After supplementation of $CdCl_2$ and plant extracts, the blood samples were incubated for 72 h at 37 °C to adjust to body conditions. Each individual whole blood culture without $CdCl_2$ or *G. glabra* extract was used as a control group. Table 1 shows the chemical composition of *G. glabra* samples according to the analysis of Khalaf et al. (2010) by using HPLC technique.

Table 1 Chemical composition of G. Glabra extracts

Compound	Amount (mg/l of extract)
Ferulic acid	5.01
p-coumaric acid	2.17
Gentisic acid	ND
Luteolin	0.33
Apigenin	1.05
Caffeic acid	ND
Sinapic acid	2.03

ND not detected

Genotoxicity testing

SCE assay

With the aim of providing successive visualization of SCEs, 5-bromo-20-deoxyuridine (Sigma®) was added at culture initiation. The cultures were incubated in complete darkness for 72 h at 37 °C. Exactly 70 h and 30 min after beginning the incubations, demecolcine (*N*-Deacetyl-*N*-methylcolchicine, Sigma®) was added to the cultures. After hypotonic treatment (0.075 M KCl), followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation, and resuspension, the cell suspension was dropped onto chilled, grease-free microscopic slides, air-dried, aged for 3 days, and then differentially stained for the inspection of the SCE rate according to the fluorescence plus Giemsa (FPG) procedure. For each treatment condition, well-spread 25 s division metaphases containing 42–46 chromosomes in each cell were scored by one observed (E. Dirican), and the values obtained were calculated as SCEs per cell.

MN assay

The MN test was performed by adding cytochalasin B (Sigma®) after 44 h of culture. At the end of the 72 h incubation period, the lymphocytes were fixed with ice-cold methanol/acetic acid (1:1, v/v). The fixed cells were put directly on slides using a cytospin, and stained with Giemsa solution. All slides were coded before scoring. The criteria for scoring MN were as described by Fenech (1993). At least 1,000 binucleated lymphocytes were examined per concentration for the presence of one, two or more MN by one observer (E. Dirican).

TAC analysis

Automated total antioxidant capacity (TAC) assays were carried out in the culture medium by commercially available kits (Rel Assay Diagnostics ®, Gaziantep, Turkey) in plasma samples obtained from blood cultures for 2 h (Erel 2004).

MTT assay

Cytotoxicity was assessed by measuring the formation of formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in a spectrophotometrically test, modified after Mosmann (1983). Blood cells were incubated with 0.7 mg/ml MTT for 24 h at 37 °C at the end of the experiment. After washing with PBS the blue formazan was extracted from cells with isopropanol/formic acid (95:5). Cytotoxicity was photometrically determined at 560 nm (Lewerenz et al. 2003).

Statistics

Statistical analysis was performed using SPSS Software (version 18.0, SPSS, Chicago, IL, USA). For statistical analysis of obtained data Duncan's test was used. Statistical decisions were made with a significance level of 0.05.

Results

SCE and MN were analyzed in human peripheral lymphocytes treated for 72 h with different concentrations of $CdCl_2$ in presence or absence of *G. Glabra* extract (Figs. 1, 2). The SCE and MN frequencies observed in cultures treated with $CdCl_2$ (5 ppm) were significantly higher than control values. But, *G. glabra* extract did not lead to genetic damages at all tested concentrations. Moreover, it was successful against $CdCl_2$-induced genotoxicity in blood cells at the applied dose. When $CdCl_2$ treated cultures were treated with *G. glabra* extract, SCEs/cell and MN/1000 cell values decreased significantly as compared to $CdCl_2$ treatment alone. Figure 3 shows the results of cytotoxicity measured by MTT assay. When assayed in vitro on human blood cells using the MTT assay, the

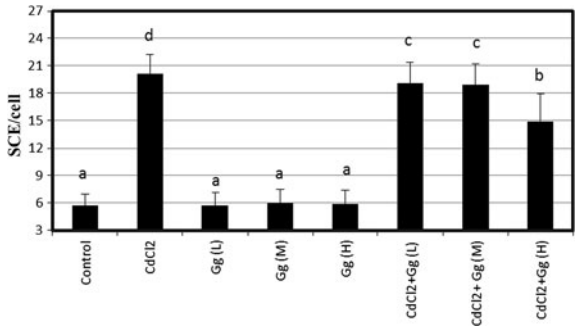

Fig. 1 Effect of *G. glabra* water extracts on cadmium chloride induced SCE formations in human peripheral lymphocytes. Values are expressed as means of five cultures in each group; means in the figure followed by the different letters present significant differences at the $P < 0.05$ level; ($CdCl_2$: Cadmium chloride; Gg (L): 5 ppm *G. glabra* extract; Gg (M): 10 ppm *G. glabra* extract; Gg (H): 20 ppm *G. glabra* extract)

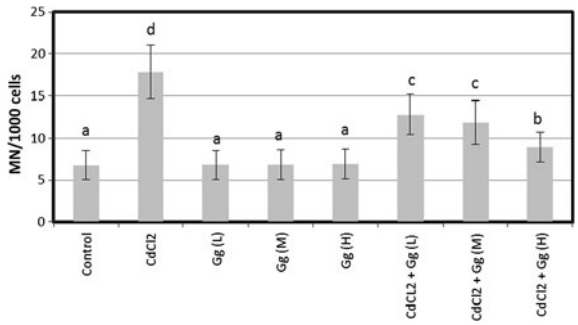

Fig. 2 The frequencies of MNs (%) in human lymphocytes treated with different concentrations of $CdCl_2$ and Gg. Abbreviations are as in Fig. 1

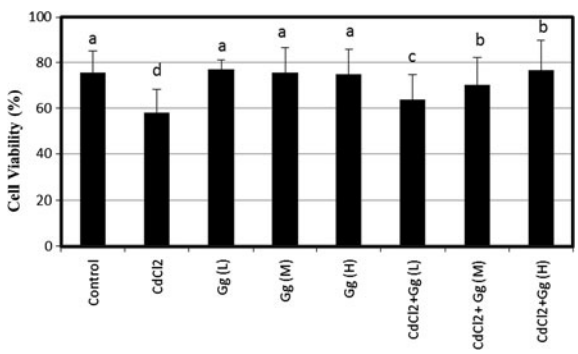

Fig. 3 3-(4,5-Dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction in human blood cultures maintained for 24 h in the presence of $CdCl_2$, Gg and their combinations. Abbreviations are as in Fig. 1

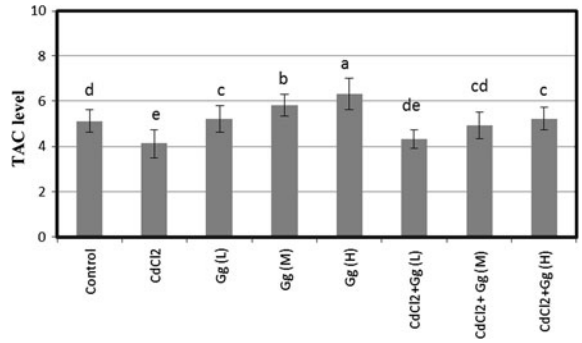

Fig. 4 Extracellular total antioxidant capacity (TAC) levels (expressed as mmol Trolox Equiv./L) in human blood cells maintained for 2 h in the presence of $CdCl_2$, Gg and their combinations. Abbreviations are as in Fig. 1

cell viability was found to be lower in $CdCl_2$ -treated cultures as compared to control group. However, the three doses of *G. glabra* extract (5, 10 and 20 ppm) improved cellular in vitro activities with respect to the cytotoxicity of $CdCl_2$. Figure 4 shows the effect of *G. glabra* extract and $CdCl_2$ on TAC levels in human whole blood cultures. As shown in Fig. 4, the TAC value decreased in comparison to the sole addition of $CdCl_2$. In contrast *G. glabra* extract increased the TAC level when applied alone to the cultures. Moreover, *G. glabra* extract had dose dependent inhibitory effects on oxidative damage caused by $CdCl_2$ in human blood cells.

Discussion

Heavy metals accumulate in the soft tissues, resulting in stress conditions, manifested by lower energy levels and damage to blood composition, kidney, lungs, liver and other vital organs. Cd compounds inhibit the repair of DNA damaged by other agents, thereby enhancing their genotoxicity (Rossman et al. 1992). Cd as the chloride salt is immediately bound to plasma proteins and subsequently accumulates primarily in liver and in other tissues; these processes do not exclude the possibility that after some time Cd accumulation was a factor in the induction of DNA damage observed in kidney (Foulkes 1994). In accordance with previous data we determined increased SCE and MN rates after exposure to $CdCl_2$ in cultured human lymphocytes. The cytotoxicity as well as genotoxicity of $CdCl_2$ is well studied. It appears that

its toxicity is mainly due to oxidative deterioration of biomolecules including DNA, proteins and lipids (Fotakis et al. 2005; Turkez 2011; Turkez et al. 2012b). Moreover, according to our results $CdCl_2$ caused oxidative stress in human blood lymphocytes, because decreased TAC levels were found in cultures only treated with $CdCl_2$ as compared to the control group. In addition, cytotoxicity, the degree to which a chemical can cause cell damage, is assessed in this study by the means of MTT assay. As shown in Fig. 3, the MTT assay results revealed that $CdCl_2$ was cytotoxic to human blood cells. Overall, $CdCl_2$ caused significant decreases of % viability of blood cells. Consistent with our finding, MTT assay demonstrated that the viabilities of hepatocyte were significantly decreased after $CdCl_2$ treatment (Wang et al. 2007).

$CdCl_2$ promotes formation of ROS such as hydrogen peroxide, which may cause cell membrane damage and DNA strand breaks (Watjen and Beyersmann 2004). Cells are normally able to defend themselves against ROS damage through the use of enzymes such as GSH, GST, SOD and catalase. $CdCl_2$ has high affinities for glutathione (GSH), which is the primary intracellular antioxidant and conjugating agent (Kidd 1997). Under normal conditions, the inherent defense system, including glutathione and the antioxidant enzymes, protects against oxidative damage. Besides, enhanced production of free radicals and inhibition of antioxidant enzymes have been suggested as possible mechanisms to explain $CdCl_2$-induced oxidative damages (Nzengue et al. 2008). ROS impairs cell membrane stability and causes cell death by lipid peroxidation (Sun 1990). Cd was found to decrease the mitotic index (MI) and also induced chromosomal abberations and micronucleus (MN) formation in plant root cells (Zhang and Yang 1994; Zhang and Xiao 1998). In several studies, it is indicated that Cd damaged the nucleolar structure, DNA and RNA in both animal and plant cells (Misra et al. 1998; Jonak et al. 2004). Some researchers reported that the Cd salts are not directly genotoxic in rodent cell lines. According to the International Agency for Research on Cancer Cd is suspected as co-mutagen in human carcinogenesis (IARC 1993). Also, Cd, a potent immunotoxic metal, induces DNA strand breaks, sister chromatid exchanges and chromosomal aberrations in human cells; on the other hand, lead is considered a potential mutagen by inducing direct DNA damage, clastogenicity and inhibition of DNA synthesis or interfering with DNA repair (Donma and Donma 2005).

Our results clearly reveal that *G. glabra* extract exhibits antigenotoxic properties at concentrations of 5, 10 and 20 ppm. Moreover, we suggest that *G. glabra* extract can be a new source of therapeutics against oxidative DNA damages as recognized in this study. *G. glabra* extract is beneficial in cases of $CdCl_2$ to inhibit blood cell damage by correcting the disturbance of oxidant/antioxidant balance system and ensures early recovery from $CdCl_2$ toxicity. Therefore, *G. glabra* extract decreases incidences of DNA damages. The root extract of *G. glabra* has antioxidant activity reportedly due to the presence of a variety of phenolic compounds including flavonoids, isoflavonoids, chalcones, and bibenzyls (Fuhrman et al. 1997). In addition, antioxidants (both enzymatic and non-enzymatic) play a central role in cellular oxidant defense systems that protect cells against damage induced by free radicals, such as superoxide anion and hydrogen peroxide (H_2O_2) (Cerutti et al. 1994; Valko et al. 2005; Turkez and Geyikoglu 2010a).

Many chemical studies revealed that licorice roots contain many saponins and flavonoids, along with glycyrrhizin (GL) (Nomura and Fukai 1998; Shibata 2000). In addition, many species-specific flavonoids, such as glabridin (GB) for *G. glabra* and glycycoumarin (GC) for *G. uralensis*, were detected in the underground parts of the respective Glycyrrhiza species (Zeng et al. 1991; Nomura and Fukai 1998). Variation in flavonoid contents in leaves of *G. glabra* was also reported (Hayashi et al. 1996; Shibano et al. 1996). Pinocembrin (PN) and licoflavanone (LF) were also isolated from the leaves of *G. glabra* (Fukui et al. 1988). The primary active constituent of Glycyrrhiza, as it relates to hepatic disorders, is the triterpene glycoside glycyrrhizin (also known as glycyrrhizic acid or glycyrrhetinic acid). Other constituents of Glycyrrhiza include flavonoids (liquiritin and isoliquiritin), isoflavonoids (isoflavonol, kumatakenin, licoricone, and glabrol), chalcones, coumarins (umbelliferone, herniarin), triterpenoids, and phytosterols. Besides, it is known that licorice contains bioactive polyphenols. *G. glabra* is a major component of many antihepatotoxic polyherbal formulations (Rajesh et al. 2000). Isoflavan derivatives glabridin, hisplaglabridin A, hisplaglabridin B and 4′- O-methyl glabridin have been isolated from *G. glabra*. These chemicals were

reported to provide protection against oxidative stress (Haraguchi et al. 2000).

In parallel to our present findings, Lee et al. (2007) reported that the association between oxidative stress and the inflammatory responses in the hepatoprotective effect of glycyrrhizin against CCl_4-induced hepatotoxicity. Chandrasekaran et al. (2011) reported that *G. glabra* is not mutagenic in a battery of genotoxicity tests. In search for lead molecules in cancer chemoprevention from natural products, a fraction 'Rlicca' isolated from *G. glabra* was studied for modulatory effects against hydrogen peroxide and 4-nitroquinoline-*N*-oxide induced genotoxicity in *Escherichia coli* PQ37 using the SOS chromotest and in human peripheral blood lymphocytes using the Comet assay (Kaur et al. 2009). The traditional medicinal properties of Glycyrrhiza include demulcent, expectorant, antitussive, and mild laxative activity (Leung 1980). Recent studies have brought to light the ability of Glycyrrhiza to enhance the detoxification of medications and toxins. In a Russian study, hepatotoxicity reactions in patients being treated for tuberculosis were significantly reduced in patients who received herbal liver support, including a combination of Glycyrrhiza, nettle (Urtica), tansy (Tanacetum), and mint (Mentha) (Galitskiĭ et al. 1997).

In conclusion, the findings of this research clearly indicated that *G. glabra* extract modulated $CdCl_2$-induced genetic damage in human blood cultures due to its antioxidant and detoxifying nature. Again, *G. glabra* can be a new resource of therapeutics against oxidative DNA damages as recognized in this study.

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