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The effect of grape seed extract on radiation-induced oxidative stress in the rat liver

Sıçan karaciğerinde radyasyonun yol açtığı oksidatif strese üzüm çekirdeği ekstresinin etkisi

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Background/aims: *The tolerance of the liver is considerably low when an effective radiation (RTx) dose needs to be delivered in patients in whom either their liver or whole body area has to be irradiated. The aim of this study was to evaluate the possible protective effect of grape seed extract on liver toxicity induced by RTx in the rat liver.* ***Methods:*** *We used four groups, each consisting of 12 healthy male Wistar rats. RTx-grape seed extract group: rats were given grape seed extract (100 mg/kg) orally for seven days, following 8 Gy whole body irradiation, and grape seed extract was maintained for four days. RTx group: the same protocol was applied in this group; however, they received distilled water instead of grape seed extract. Grape seed extract group: only grape seed extract solution was administered for 11 consecutive days in the same fashion. Control group: only distilled water (orally) was administered in a similar manner. The level of malondialdehyde, an end product of lipid peroxidation, and the activities of superoxide dismutase and catalase, two important endogenous antioxidants, were evaluated in tissue homogenates.* ***Results:*** *Grape seed extract was seen to protect the cellular membrane from oxidative damage and consequently from protein and lipid oxidation. In the RTx group, malondialdehyde levels were extremely higher than those of the grape seed extract-RTx group ($p<0.001$). Grape seed extract administration moderately reserved the malondialdehyde levels. RTx therapy decreased superoxide dismutase and catalase activities in the liver homogenates ($p<0.001$), and these alterations were significantly reversed by grape seed extract treatment ($p<0.001$). There were no differences between the grape seed extract-RTx, grape seed extract and control groups with regard to antioxidant activity ($p>0.05$).* ***Conclusions:*** *The levels of antioxidant parameters on RTx-induced liver toxicity were restored to control values with grape seed extract therapy. Grape seed extract may be promising as a therapeutic option in RTx-induced oxidative stress in the rat liver.*

Key words: Grape seed extract, radiation, oxidative stress

Amaç: *Tüm vücut ışınlaması yapılması veya karaciğere radyoterapi verilmesi gereken hastalarda, önerilen etkin dozlarda karaciğerin toleransı oldukça düşüktür. Bu çalışmanın amacı, sıçan karaciğerinde radyasyonun (RTx) neden olduğu toksisite üzerine üzüm çekirdeği ekstresinin oluşturabileceği muhtemel koruyucu etkiyi değerlendirmektir.* ***Yöntem:*** *Her biri sağlıklı, erkek, on iki Wistar sıçandan oluşan dört grup oluşturuldu. RTx-üzüm çekirdeği ekstresi grubu; yedi gün oral üzüm çekirdeği ekstresi (100 mg/kg) ardından 8 Gy tüm vücut ışınlaması yapıldı ve üzüm çekirdeği ekstresi tedavisine 4 gün daha devam edildi. RTx grubu; aynı işlemler uygulandı, ancak üzüm çekirdeği ekstresi yerine oral distile su verildi. Üzüm çekirdeği ekstresi grubu; sadece üzüm çekirdeği ekstresi solüsyonu aynı tarzda 11 gün boyunca verildi. Kontrol grubu; sadece distile su aynı şekilde verildi. Lipit peroksidasyonu son ürünü malondialdehid düzeyi ve iki önemli endojen antioksidan olan süperoksid dismutaz ve katalaz aktivitesi karaciğer doku homojenatlarında çalışıldı.* ***Sonuç:*** *Üzüm çekirdeği ekstresi hücre membranında protein ve lipit peroksidasyonunu engelledi ve takiben oksidatif hasarı geçirdi. RTx grubunda malondialdehid seviyesi; RTx-üzüm çekirdeği ekstresi grubundan belirgin şekilde daha yüksekti ($P<0.001$). Üzüm çekirdeği ekstresi ilavesiyle malondialdehid seviyesinde orta derecede azalma gözlendi ($P<0.001$). RTx uygulaması karaciğer homojenatlarında süperoksid dismutaz ve katalaz aktivitesini azaltırken ($P<0.001$), üzüm çekirdeği ekstresi tedavisi ile bu değişiklikler belirgin derecede düzeldi ($P<0.001$). Antioksidan aktivite açısından RTx-üzüm çekirdeği ekstresi grubu ile üzüm çekirdeği ekstresi ve kontrol grubu arasında herhangi bir fark gözlenmedi ($P<0,05$).* ***Tartışma:*** *Radyasyonun neden olduğu karaciğer toksisitesinde, antioksidan parametrelerin seviyeleri üzüm çekirdeği ekstresi uygulaması ile kontrol değerlere ulaştı. Üzüm çekirdeği ekstresi radyoterapinin sıçan karaciğerinde yol açtığı oksidatif stresi azaltmada bir tedavi ümidi olabilir.*

Anahtar kelimeler: Üzüm çekirdeği ekstresi, radyasyon, oksidatif stres

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INTRODUCTION

Antioxidants that accumulate in liver tissue are potential candidates for prevention or treatment of disorders involving oxidative damage. Animal models have provided a wealth of information on the biological effects of photochemicals from vegetables and fruits on the oxidative damage during radiochemotherapy. Grape seeds are rich sources of monomeric phenolic compounds such as catechin, epicatechin and dimeric, trimeric and tetrameric proanthocyanidins (1). These molecules possess a structure that confers on them an antioxidant property, which has been demonstrated to exert a novel spectrum of biological, pharmacological, therapeutic, and chemoprotective effects against oxygen free radicals and oxidative stress (2). Grape seed extract (GSE) has a protective effect on oxidant-induced production and deposition of extracellular matrix components, which results in hepatic fibrosis (3). It also improves hepatic ischemia-reperfusion injury and reduces the size of the infarct in cardiac ischemia in the rat (4, 5). Several studies have indicated that extracts obtained from grape seed inhibit enzyme systems that are responsible for the production of free radicals, and that they are antimutagenic and anticarcinogenic (6,7). For this reason, GSE is widely consumed as a dietary supplement and could be useful in synergizing the efficacy of chemotherapeutic agents in cancer treatment.

The most common cancer treatment modality promising a cure appeared to be a combination of radiotherapy and chemotherapy. Following radiotherapy, the risk of normal tissue complication constitutes a significant clinical concern and limits the radiation (RTx) dose that can be delivered to the patients (8). The killing action of RTx (X-rays, γ-rays) is mainly mediated through the free radicals generated from the radiolytic decomposition of cellular water. When these free radical species interact with critical targets such as DNA and membranes, irreversible damage occurs, leading to cell death. Cell survival and adaptation to an environment containing RTx can mainly depend on the ability of cells to maintain optimal function in response to free radical-induced damage at the biochemical level. Since biological antioxidants inactivate free radicals and their products, the enzymes involved in the metabolism of reactive oxygen species (ROS) are expected to play important roles in the radiosensitivity of cells. Increased oxidation after exposure to RTx has been observed in numerous studies, as well as in different tissues (9). Although RTx therapy is a common and important tool for cancer treatment, the radiosensitivity of normal tissues adjacent to the tumor limits the therapeutic gain. The response of normal tissues to therapeutic RTx exposure ranges from those that cause mild discomfort, to others that are life-threatening. The speed at which a response develops varies widely from one tissue to another and often depends on the dose of RTx that the tissue receives (10).

The combination of chemotherapy and RTx in particular produces hepatic toxicity when RTx is used in the treatment of intrahepatic tumors, lymphomas, ovarian cancers, and bone marrow transplantation (11). Radiation-induced liver disease (RILD) is a dose-limiting complication of liver RTx limiting the options for treatment of RILD, and in severe cases, liver failure and death can occur (12). Considerable efforts are being devoted at present to improvement of radiotherapy. One of the main ways in which such an improvement may be obtained is by scavenging oxidant products (13). The effects of RTx exposure that become apparent to the patient in the course of weeks, months or years after radiotherapy, are seen both in the tumor and in normal tissues that surround a tumor, which are unavoidably exposed to RTx. Oxidative damage is considered to be one of the most popular and important effects of radiotherapy in the liver. Considerable work has been carried out on GSE against free radical-associated tissue injury, but its effect and role in RILD remain to be elucidated. Therefore, in the present study, we investigated the role of GSE against RTx-induced oxidative stress in the rat liver.

MATERIALS AND METHODS

Animals

Forty-eight male Wistar rats, purchased from the Animal House of the Faculty of Medicine, Erciyes University, and weighing 240–320 g, were included in the study. The experimental protocol used was approved by the Department of Animal Care and Use Committee of the Turkish Ministry of Agriculture, and adhered to the European Community Guiding Principles for the Care and Use of Animals. The animals were fed a standard rat chow diet, had access to water ad libitum, and were synchronized by the maintenance of controlled environmental conditions (light, temperature, feeding time, etc.) for at least two weeks prior to and throughout the experiments.

Experimental Design

The animals were divided into four groups, each consisting of 12 animals. RTx-GSE group: GSE (100 mg/kg) solution was administered for seven consecutive days by a curved 16-gauge gavage tube inserted after applying a proper restraint. This group received irradiation as 8 Gy whole body irradiation, and GSE was maintained for four additional days. RTx group: the same protocol was applied to this group except that they received distilled water instead of GSE in a similar manner (volume equal to that used for extract administration in experimental animals) along with procedure. GSE group: only GSE solution was administered for 11 consecutive days in the same fashion. In the remaining rats (control group), only distilled water was administered orally.

Whole body irradiation

In order to immobilize the animals, mild hypnosis was induced by intramuscular administration of ketamine (50 mg/kg), 5 minutes before RTx, ensuring spontaneous respiration throughout the procedure. The animals were then paired and placed in supine position on a Plexiglass board, so that two animals would be irradiated at a time. Rats of the groups were exposed to a single dose of 8 Gy whole body irradiation of gamma RTx from a ^{60}Co source (Theratron 780 C), at a dose rate of 0.52 Gy/min, administered at 2 cm depth below the skin, the source-skin distance being 80 cm (14).

Preparation of homogenates

Following their exposure to RTx, the animals were placed individually into metabolic cages. After a 96-hour interval, all rats were decapitated under mild anesthesia (we used 50 mg/kg, i.p. ketamine). Blood samples were collected from each rat and complete blood counts were analyzed. Livers were excised immediately and were homogenized in 10-fold volume of phosphate buffer solution, pH 7.4, by using a homogenizer (Ultra-Turrax T 25, IKA; Werke 24,000 r.p.m.j. Germany). The homogenates were centrifuged at 10 000xg for about 60 min and the resulting supernatants were stored at -80°C until the time for malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) assays. Tissue protein was identified using the Lowry method (15). All reagents were purchased from Sigma (Sigma-Aldrich Corp, St. Louis, MO, USA).

Preparation of grape seed extract

Ripened grapes (Vitis vinifera L) of the most popular wine-making grape cultivars grown in Turkey, Öküzgozü (red grape cultivar), were obtained from the Tokat region in Turkey. After harvest, undamaged and disease-free berries were snipped from clusters. Following manual separation of the seeds from whole berries, seeds and bagasse (berry without seed and juice) were dried at 70°C for 72 hours, separately. Dried grape seeds were ground to fine powder with a grinder. Then the powdered grape seeds (100 g) were extracted in a Soxhlet extractor with petroleum ether (60°C for 6 h) to remove the fatty materials. The defatted grape seed powder was re-extracted in a Soxhlet apparatus for 8 h with 200 ml ethanol. After that, the extract was concentrated in a rotary evaporator (Rotavator Evaporator R 200, Buchi, Switzerland) under vacuum at <40°C to get crude extracts, was lyophilized (Labconco Freezone 2.5, Missouri, USA), and the extract was then stored in a dark bottle until use at 4°C (16).

The concentration of total phenolic compounds in the seed extract was determined by the Folin-Ciocalteu colorimetric method of Singleton and Rossi (17). Estimations were carried out in triplicate and calculated from a calibration curve obtained with gallic acid. Total phenolics were expressed as gallic acid equivalents (milligram GAE per gram extract). The content of the total phenolics was found to be 573.5±6.80 mg GAE per gram in the GSE. Gokturk-Baydar et al. (18) and Jayaprakasha et al. (19) had also reported similar results.

Determination of MDA level

The levels of MDA in liver tissue were assessed according to the method of Ohkawa et al. (20). The assay procedure for the MDA level in the rat liver was set up as follows: to samples less than 0.2 ml of 10% (w/v) tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulphate and 1.5 ml of 20% acetic acid solution were added. pH was adjusted to 3.5 with NaOH and 1.5 ml of 0.8% aqueous solution of TBA. The final volume was brought to 4.0 ml with distilled water and then heated in an oil bath at 95°C for 60 min using a glass ball as a condenser. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) were added and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was then obtained and its absorbance was measured at 532 nm. MDA levels were expressed in nanomoles MDA per milligram of protein in tissue homogenates (nmol/mg protein).

Determination of SOD activity

The SOD activity of liver tissue was determined according to the method of Sun et al. (21). The principle of the method is based on inhibition of nitro blue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. The SOD activity was expressed as U/mg protein.

Determination of CAT activity

CAT activity was determined in the homogenate as described by Aebi (22). Briefly, 100 µl of the tissue supernatant was incubated with an equal volume of absolute alcohol for 30 min at 0°C, followed by the addition of triton X-100. A known volume of this tissue reaction mixture was taken in an equal volume of 0.066 M hydrogen peroxidase (H_2O_2) in phosphate buffer and absorbance was measured at 240 nm for 30 s in a spectrophotometer. An extinction coefficient of 43.6 M/cm was used to determine the enzyme activity, which was expressed in terms of mM of H_2O_2 degraded/min/mg protein.

Statistical Analysis

Data were expressed as mean ± standard deviation *($\bar{x}$±SD)*. Comparison of SOD, CAT and MDA between the groups was made using the One Way Analysis of Variance (ANOVA). Post-hoc comparisons on parameters were performed using the Tukey procedure. Statistical significance was set at $p<0.05$. All analyses were performed with the statistical package for scientist (SIGMASTAT) Windows version 3.10.

RESULTS

As we expected, RTx decreased the white blood cell count (WBC), red blood cell count (RBC), hematocrit (HCT), and lymphocyte count (LYM), and increased the mean cell hemoglobin (MCH) and the mean cell hemoglobin concentration (MCHC) in rats. Table 1 shows that hematological analysis of the groups. GSE treatment did not affect the hematological parameters. With regard to complete blood counts, there was no difference between GSE-only group and the control group.

A highly significant increase in MDA levels ($p<0.001$) and decreases in the activities of SOD and CAT ($p<0.001$) in the liver homogenates were indicated as a result of RTx exposure when compared with the non-irradiated rat groups (Figures 1-3). When comparing the groups receiving RTx, GSE treatment reversed MDA, SOD and CAT values to near control levels ($p<0.001$). There were no differences between GSE-only and control group. Table 2 shows the effect of GSE on oxidative

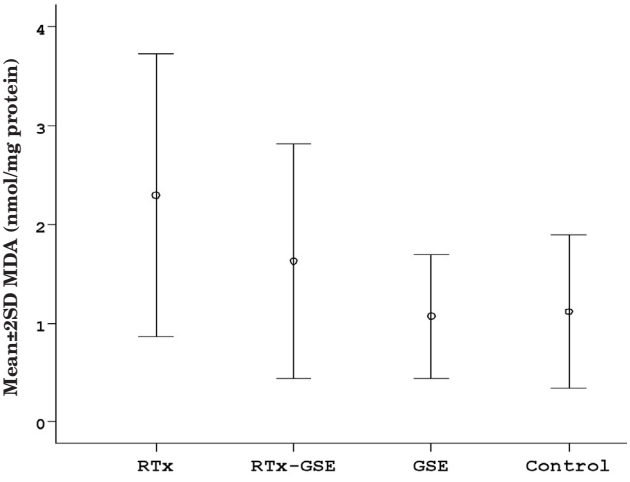

Figure 1. MDA activity in the RTx group was significantly higher than in RTx-GSE, GSE and control groups ($p<0.001$). There were no significant differences between RTx-GSE, GSE and control groups ($p>0.05$).

Table 1. Hematological analyses of the groups

Variables	RTx n=12 $\bar{X}$±SD	RTx-GSE n=12 $\bar{X}$±SD	GSE n=12 $\bar{X}$±SD	Control n=12 $\bar{X}$±SD	p
WBC (cells/µL)	2.18±0.67[a]	1.77±0.59[a]	5.83±0.93[b]	6.00±1.08[b]	<0.001
RBC (x10⁶cells/µL)	6.48±0.82[a]	6.38±0.96[a]	7.30±0.58[b]	7.30±0.59[b]	0.003
HGB (g/100 ml)	13.19±1.47	13.00±1.77	13.92±0.91	14.04±0.93	0.154
HCT (%)	40.87±5.02[ab]	39.36±5.45[a]	44.51±51[b]	44.76±2.34[b]	0.003
MCV (fl)	63.15±2.26	62.58±2.57	63.28±2.84	61.26±2.58	0.215
MCH (pg)	20.39±0.73[a]	20.48±0.81[a]	19.49±1.40[ab]	19.21±1.53[b]	0.022
MCHC (g/dl)	32.33±0.74[ab]	33.07±0.99[a]	31.47±1.97[ab]	31.21±1.80[b]	0.013
PLT (x10³ cells/µL)	592.17±263.60	663.33±195.67	663.75±417.72	711.92±390.01	0.847
LYM (cells/µL)	1.60±0.48[a]	1.08±0.35[a]	4.98±0.94[a]	4.92±0.98[b]	<0.001

WBC: Total white blood cells. RBC: Red blood cell count. HGB: Hemoglobin. HCT: Hematocrit. MCV: Mean cell volume. MCH: Mean cell hemoglobin. MCHC: Mean cell hemoglobin concentration. PLT: Platelet count. LYM: Lymphocyte.

Table 2. Oxidative stress parameters of the groups

Variables	RTx n=12 $\overline{X}$±SD	RTx-GSE n=12 $\overline{X}$±SD	GSE n=12 $\overline{X}$±SD	Control n=12 $\overline{X}$±SD	P*
MDA	2.29±0.72[a]	1.63±0.59[b]	1.07±0.31[b]	1.12±0.39[b]	<0.001
SOD	1.08±0.09[a]	1,79±0.16[b]	1.70±0.19[b]	1.63±0.13[b]	<0.001
CAT	125.32±15.50[a]	162.33±14.52[b]	165.53±13.60[b]	156.41±16.86[b]	<0.001

*ANOVA test to compare SOD, CAT and MDA among groups; statistically significant (p<0.05) differences SOD, CAT and MDA between groups are labeled with different letters; Power of performed test with alpha for SOD, CAT and MDA = 0.050: 1.000
MDA: Malondialdehyde. SOD: Superoxide dismutase. CAT: Catalase.

stress parameters in rat liver exposure to radiotherapy.

DISCUSSION

Radiation is known to induce oxidative stress through generation of ROS, resulting in imbalance of pro-oxidants and antioxidants in the cells, which is suggested to culminate in cell death (23, 24). Radiation produces disruption of sensitive molecules in the cells, whereas the other actions of RTx occur when it interacts with water molecules in the cell, resulting in the production of highly reactive free radicals, such as $^{\cdot}OH$, $^{\cdot}H$, and e^{-}_{aq}. High energy RTx breaks the chemicals bonds and this creates free radicals, such as those produced by other insults, as well as by normal cellular processes in the body. The free radicals can change the chemicals in the body (13). The most important of the early effects of RTx is that it produces ROS. These species can induce the cellular antioxidant defense enzymes such as SOD, glutathione peroxidase (GSH) and CAT (25). Intracellular generation and accumulation of ROS, such as the superoxide anion, hydrogen peroxide, singlet oxygen, and the hydroxyl radical, in the stressed cells overcome the natural antioxidant defense, causing damage to biological macromolecules, including nucleic acids, proteins and lipids.

The involvement of free radical scavengers in protecting against RTx damage was highlighted when scientists found that whole body RTx decreased the total antioxidant capacity of organisms and that the levels of known antioxidants such as ascorbic acid and uric acid were depleted. The ability of certain substances to provide protection against the damaging effects of RTx was first published in 1949 (26). The best-known radioprotectors are the sulfhydryl compound products, such as cysteine and cysteamine (27). To date, the most effective compound of this type, originally tested against lethal doses of X-rays and γ rays in mice, is WR-2721, the common name of which is amifostine (28). Because of limited success achieved over the years in testing the radioprotective efficacy of a

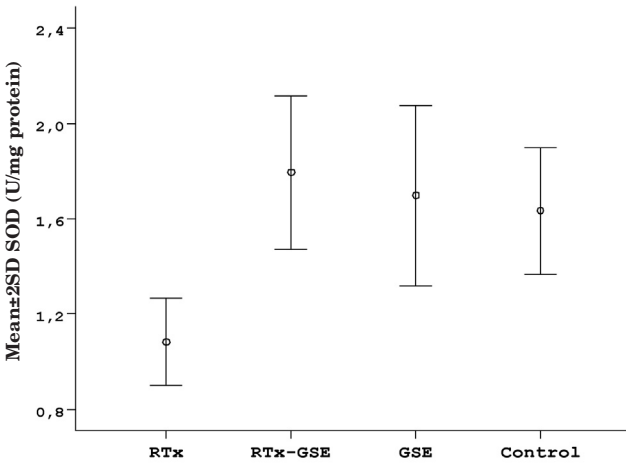

Figure 2. SOD activity in the RTx group was significantly lower than in RTx-GSE, GSE and control groups (p<0.001). There were no significant differences between RTx-GSE, GSE and control groups (p>0.05).

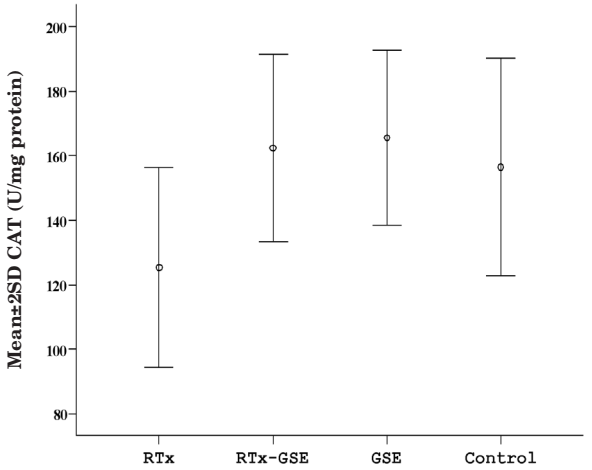

Figure 3. CAT activity in RTx group was significantly lower than in RTx-GSE, GSE and control groups (p<0.001). There were no significant differences between RTx-GSE, GSE and control groups (p>0.05).

number of compounds, there is still an urgent need to identify novel, nontoxic, effective, and convenient compounds to protect humans from the damaging effects of RTx.

In the present study, we investigated whether or not there were any beneficial effects of GSE on RTx-induced oxidative stress in the rat liver. Several authors have reported that RTx is a useful compound for the study of oxidative stress, because its effects and toxicity are mediated by free radicals (29, 30). Furthermore, antioxidant substances, such as melatonin and amifostine, have been suggested to play a role in the protection against RTx-induced toxicity (31, 32). The effect of RTx on the rat liver or on isolated rat hepatocytes has been documented in previous studies (33). In our study, the exposure of whole rat bodies and hepatocytes to RTx resulted in an increased oxidative damage in the radiotherapy-only group.

GSE-containing flavonoids are currently used as nutritional supplements. In addition to their antioxidant benefits, seed extracts have been shown to exert chemo-preventive and anticancer effects (34-36). GSE is an extract by-product obtained from the grape seed and it contains a variety of biologically active species used for protection against oxidative stress induced by free radicals and ROS (37). According to its chemical composition, GSE may have a digestive behavior similar to that of other grape by-products. In relation to their polyphenol compounds, as shown by our results, GSE contains mainly flavonoids, all involved in ameliorating the oxidative stress *in vitro* and *in vivo* (38, 39). To evaluate their potential as antioxidants, we have studied the effect of the extract on oxidative damage and on antioxidant defense of hepatocytes exposed to oxidative stress.

Our results have shown that oral intake of GSE reduces the oxidative effects of RTx on the rat liver. In fact, we detected low MDA levels in rats receiving GSE. GSE prevented the antioxidant consumption of hepatocytes in rats exposed to RTx. In this study, GSE reversed SOD and CAT activity values approaching those of the control group. A decrease in the levels of antioxidants in the hepatocytes indicates an enhancement in peroxidation, leading to a loss of membrane integrity and oxidative modifications of amino acid side chains, etc. (40). GSE treatment considerably increased the formation of antioxidants products in hepatocytes, and this effect may be due to the phenolic composition of GSE and its antioxidant activity.

Flavonoids may also exert antioxidant abilities through protection or enhancement of endogenous antioxidants. SOD and CAT scavenge the free radicals activated by RTx. The conjugation of RTx and antioxidant enzymes advances the detoxification of RTx. The concentrations of cellular thiols, such as GSH, play an important role in the maintenance of cellular redox state. Numerous flavonoids have been shown to alleviate the oxidative stress by increasing the endogenous antioxidant status, protecting cells against free-radical damage by increasing resistance to oxidative stress (41, 42). In agreement with previous reports, we found that SOD and CAT levels were diminished in RTx-exposed rat hepatocytes (31), while the combination with GSE restored the level of antioxidants.

These data lead to the conclusion that oxidative stress is one of the mechanisms of RTx cytotoxicity and that GSE has protective effects against such oxidative damage. In this context, recent reports show that naturally occurring dietary supplements with known anti-cancer activity could be used in combination with radiotherapy to reduce the toxicity produced by RTx (43). Therefore, based on the data shown in the present study, GSE could be useful in synergizing the cancer therapeutic efficacy of RTx treatment. Further studies are needed to ensure GSE efficacy in humans.

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