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Original research paper

Effect of selected catechins on doxorubicin antiproliferative efficacy and hepatotoxicity *in vitro*

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Catechins may influence both desirable and undesirable effects of many drugs. In this study, the *in vitro* effect of (+)-catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate, and (–)-epigallocatechin gallate (EGCG) on the efficacy of anticancer drug doxorubicin (DOX) was studied in HCT-8 cancer cells. Rat hepatocytes were used to study the influence of EGCG on DOX hepatotoxicity. Cell proliferation and viability were studied by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide and neutral red uptake test assays. Formation of reactive oxygen species (ROS) was determined using the dichlorofluorescein assay. All of the studied catechins (1–25 μmol L^{-1}) had no effect on the proliferation of intestinal cancer cells and did not affect the antiproliferative effect of DOX (1–8 μmol L^{-1}) in these cells. Moreover, EGCG at 25 μmol L^{-1} increased the viability of isolated hepatocytes and significantly protected these cells against DOX-induced toxicity and ROS production. Consumption of EGCG during DOX therapy seems to be safe and beneficial, since EGCG does not decrease DOX anticancer efficacy and could ameliorate DOX hepatotoxicity.

Keywords: epigallocatechin gallate, chemoprevention, HCT-8 cells, hepatocytes, doxorubicin

The anthracycline antibiotic doxorubicin (DOX) is one of the most useful anticancer agents and is still a cornerstone in the therapy of many carcinoma types. Unfortunately, DOX therapy is mostly accompanied by severe side effects based on its systemic toxicity, especially cardiotoxicity (1–3). DOX hepatotoxicity, sometimes passing into liver cirrhosis, has been also reported (4, 5).

Oxidative damage mediated by generation of reactive oxygen species (ROS), namely, induction of hydroxyl radicals formation, is most likely the mechanism of DOX antitumor activity (3, 6, 7). Quinone-containing DOX is enzymatically activated through

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one-electron reduction, by NADPH-cytochrome P450 reductase or NADH-dehydrogenase, to free radical intermediates, with subsequent production of reactive oxygen species in the presence of molecular oxygen. DOX also acts as a powerful iron chelator and the resulting DOX-Fe^{2+} complex is an efficient catalyst of the hydrogen peroxide conversion to the hydroxyl radical (8). It has been proposed that especially hydroxyl radicals may react with unsaturated lipids initiating lipid peroxidation, with cellular DNA inducing DNA damage, and oxidize certain functional proteins. Nevertheless, DOX treatment can also place non-cancerous tissues under conditions of oxidative stress and thereby impair the health of organs such as heart, liver, kidney, and brain. Such damage not only limits the effective dosage of DOX but also deteriorates the quality of life of cancer patients after chemotherapy (9, 10). Therefore, an antioxidant co-administered with cytostatics may actually reduce the severity of the adverse effects of chemotherapy. In order to eliminate DOX-mediated oxidative stress, addition of certain antioxidants to DOX therapy has been intensively studied. Many studies have focused on natural antioxidant compounds, like polyphenols (reviewed, *e.g.*, in refs. 11, 12). On the other hand, antioxidants might also reduce ROS created by cytostatics in cancer cells, and thereby interfere with the drug's antineoplastic activity (13, 14). With respect to both points of view, concurrent administration of antioxidants with DOX is questionable and further studies are necessary.

Polyphenolic compounds, ubiquitously found in fruits and vegetables, exhibit multiple biological effects, including antiviral, antibacterial, anti-inflammatory, vasodilatory, antioxidant, and antiradical activities (15). They also exert chemopreventive effects and could be used for attenuation of the side effects of cytostatics, including DOX (11).

Among the polyphenols tested, catechins showed a great potential of preventing DOX-mediated toxicity. Catechins, the main polyphenolic compounds of green tea, are considered to be very beneficial for the human organism owing to their anticancer, antibacterial, antiviral, antioxidant, and antiradical properties. The ability of catechins to attenuate DOX toxicity was frequently described. In rat, individual catechins and/or green tea extract exhibited protective effects against DOX-induced cardiovascular abnormalities (16), cardiomyocyte injury (17, 18), brain toxicity (19), and spermatogenic disorders (20). Moreover, catechins are able to inhibit carbonyl reductase 1, the main DOX deactivation enzyme (21), and in this way they enhanced DOX efficacy in tumor-bearing mice (22). On the other hand, antioxidant properties of catechins could decrease DOX--mediated oxidative stress in cancer cells, which is believed to contribute to DOX antiproliferative effect (8, 12). Owing to this fact, the risk of the possible decrease of DOX anticancer efficacy by catechins should be also kept in mind.

Nowadays, the use of antioxidants in combination with chemotherapeutic agents is an important issue in cancer treatment and it has been the subject of extensive recent debates. The mechanism of hepatoprotective activity of green tea catechins in the model of DOX-induced toxicity in non-cancerous tissues has not been elucidated yet. Therefore, the present study was designed to evaluate the *in vitro* effects of several catechins (namely, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate and (–)-epigallocatechin gallate), representing the most abundant flavonoids in green tea, on the efficacy and toxicity of DOX. For this purpose, the intestinal tumor cell line HCT-8 and primary culture of isolated rat hepatocytes were used as model systems.

EXPERIMENTAL

Chemicals and reagents

(+)-Catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechingallate and (–)-epigallocatechin gallate (EGCG), RPMI-1640 medium, Ham-F12 medium, William´s E medium, *N*-2-hydroxyethylpiperazine-*N*´-2-ethanesulfonic acid (HEPES) buffer, ethylene glycol-*bis*(aminoethylether)-*N*,*N*,*N*´,*N*´-tetraacetic acid (EGTA), 2´,7´-dichlorodihydrofluorescein diacetate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), neutral red, and collagenase from *Clostridium histolyticum* were supplied by Sigma-Aldrich (Czech Republic). Fetal bovine serum (FBS), horse serum (HS), and gentamicin sulfate were purchased from Invitrogen (USA) and bovine serum albumin (BSA) from Fluka (Czech Republic). Doxorubicin was provided by Pharmacia & Upjohn (Italy). All other chemicals were of HPLC or analytical grade.

Stock solutions were prepared in dimethylsulfoxide (DMSO) and stored at 4 °C in the dark.

Cancer cell culture

Human colorectal adenocarcinoma cell line HCT-8 was purchased from ATCC (American Type Culture Collection, USA). Cells were multiplied in three passages, frozen in aliquots and stored in liquid nitrogen. The absence of mycoplasma in all cell lines used in the laboratory was periodically checked by Generi Biotech (Czech Republic). For every set of experiments (lasting 3–9 weeks), new storage cells were resuscitated. The human colorectal adenocarcinoma cell line HCT-8 was maintained in RPMI-1640 medium supplemented with 5 % heat-inactivated FBS, 5 % heat-inactivated HS, 1 % Na-pyruvate and 0.5 % penicillin/streptomycin. Cells were grown in T-75 cm^2 culture flasks in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Preparation of primary culture of rat hepatocytes

Male Wistar rats were obtained from MediTox (Czech Republic). They were housed in air-conditioned animal quarters with a 12 h light/dark cycle. Food (a standard rat chow diet) and water were provided *ad libitum*. The rats were cared for and used in accordance with the Guide for the Care and Use of Laboratory Animals and all animal experimental procedures were approwed by the Ethical Committee of Charles University (Prague, Czech Republic). Animals were anesthetized with ether, sacrificed by decapitation, liver tissues were removed immediately and hepatocytes were prepared by the two-step collagenase method (23). Briefly, liver was perfused with salt solution [140 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 0.8 mmol L^{-1} $MgSO_4$ in Na^+/K^+ phosphate buffer (0.2 mmol L^{-1}, pH 7.4) containing a calcium binding component (0.4 mmol L^{-1} EGTA)]. Consequently, the liver was perfused with phosphate buffer containing calcium chloride (1.46 mmol L^{-1}) and collagenase (30 mg per 100 mL) at 37 °C. Collagenase perfusion proceeded for 5–6 min. After perfusion, the liver was transferred to the medium containing BSA and the hepatocytes were released. The obtained suspension was filtered through a nylon mesh and centrifuged at 40x*g* for 5 min at 4 °C. The pellet was re-suspended in

chilled buffer and the washing procedure was repeated twice. Finally, three million viable cells (75–80 %) in 3 mL of culture medium ISOM (1:1 mixture of Ham F12 and Williams' E) were placed into 96-well plates. The FBS was added to the culture medium (10 %). Cultures were maintained without the tested compounds for 4 hours at 37 °C in a humid atmosphere of air and 5 % CO_2.

Cell viability tests

Catechins were dissolved in DMSO to obtain stock solutions with concentrations of 1, 5, 10 and 25 mmol L^{-1}. In order to prepare samples with final concentrations of 1, 5, 10 and 25 µmol L^{-1}, these stock solutions were diluted 1000-times with an appropriate cell culture medium (ISOM or RPMI-1640). Hence, the final concentration of DMSO in all samples was 0.1 %. The hepatocytes or HCT-8 cancer cells cultured in the appropriate medium with 0.1 % DMSO only were used as control samples in all the tests described below.

Proliferation of cancer cells was assayed using the neutral red uptake test (NRU) after 24- and 48-h exposures. The viability of hepatocytes was tested using the MTT assay after 24-h exposure and was monitored microscopically.

NRU test. – NRU test represents the simplest and cheapest method for detection of cell viability/proliferation. The cells were cultured in 96-well plates in the RPMI-1640 medium supplemented with 5 % heat-inactivated FBS, 5 % heat-inactivated HS, 1 % Na-pyruvate and 0.5 % penicillin/streptomycin with individual catechins (concentrations 1–25 µmol L^{-1}), with DOX alone (concentrations 1–8 µmol L^{-1}) and with combinations of DOX (concentrations 1–8 µmol L^{-1}) + catechins (fixed concentration 5 µmol L^{-1}). After 24- and 48-h exposure intervals, the medium was removed and 100 µL of neutral red-containing medium was added into each well. Plates were incubated for additional 3h at 37 °C. Cells were then washed with 100 µL of PBS. Cells were fixed in a solution of 0.5 % formaldehyde/1 % calcium chloride for 15 min. The neutral red dye was extracted from viable cells with the solvent (50 % ethanol/1 % acetic acid) by shaking for 30 min at room temperature. The absorbance of solubilized dye was measured using an Infinite M200 spectrophotometer (Tecan, Switzerland) at 540 nm. Each sample was assayed in six parallels and three independent experiments were performed. Viabilities of treated cells were expressed as percentage of untreated control (100 %).

MTT assay. – For cells with weaker attachment to the plastic bottom (*i.e.*, isolated hepatocytes), the MTT assay is preferred to the NRU test as the washing steps are not required in the MTT assay. Hepatocytes were cultured in 96-well plates in the medium with epigallocatechin gallate at concentrations of 5–25 µmol L^{-1}. After exposure, 25 µL of MTT solution (3 mg of MTT in 1 mL of phosphate buffered saline, pH 7.4) was added to each well. Final MTT concentration in all wells was 1.8 mmol L^{-1}. Plates were incubated for additional 2 h at 37 °C, then the medium was removed and the formed formazan was dissolved in 50 µL of 80 mmol L^{-1} HCl in isopropanol by 30-min shaking. The absorbance in each well was quantified by measuring at 570 nm, with background correction at 690 nm. Cell-free blank samples containing EGCG in the medium only were incubated with MTT. Since no changes in the absorbance corresponding to MTT were observed, it can be concluded that EGCG did not cause reduction of MTT to formazan in this experimental setup.

Microscopic monitoring. – Hepatocytes were cultured in 96-well plates in the medium with DOX alone (concentrations 10 and 25 μmol L^{-1}) and with combinations of DOX (concentrations 10 and 25 μmol L^{-1}) + EGCG (fixed concentration 5 μmol L^{-1}). During 24-h exposure, the morphology of cells was monitored and documented using a Nicon Eclipse TS100 inverted microscope, 10x Nikon air objective, digital cool camera 1300Q (VDS Vosskühler, Germany) and software NIS-Elements AR 2.20 (Laboratory Imaging, Prague, Czech Republic).

Determination of cellular reactive oxygen species (ROS) formation

To assess ROS generation in hepatocytes, measurement of 2',7'-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) oxidation was used. This non-fluorescent reagent diffuses passively through the plasma membrane into the cell, where acetate groups are cleaved by intracellular esterases. The compound can then be oxidized by ROS formed within the cell (particularly by hydroxyl radicals, OH) to form fluorescent dichlorofluorescein, while the fluorescence intensity is proportional to the ROS level (24).

Isolated hepatocytes seeded in a 96-well plate at a density of 2,500 cells per well were washed with PBS buffer and loaded with 100 μL of 5 μmol L^{-1} H_2DCF-DA and 100 μL of PBS buffer with DOX alone (concentrations 10 and 25 μmol L^{-1}) and with combinations of DOX (10 and 25 μmol L^{-1}) + EGCG (5, 10 and 25 μmol L^{-1}). After 12 h of incubation, fluorescence intensity was measured for 60 min at 37 °C using a microplate reader (Tecan Infinite M200) at λ_{ex} = 485 nm and λ_{em} = 525 nm. The 3 % H_2O_2 solution was used as a positive control.

Statistical analysis

All calculations were done using Microsoft Excel and GraphPad Prism 6.02 (GraphPad Software, USA). Statistical significance was tested by one-way ANOVA and differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Antiproliferative effect of catechins in HCT-8 cells

The results showed no effect of the (+)-catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechingallate and (–)-epigallocatechin gallate on the viability of HCT-8 cells (Fig. 1). Even the highest concentration of studied catechins (25 μmol L^{-1}) did not significantly decrease proliferation of these cancer cells.

In contrast to our results, an antiproliferative effect of certain catechins on cancer cells was reported. Seeram *et al.* (25) observed pronounced inhibition of proliferation of MCF-7 (breast), HCT-116 (colon) and NCI-H460 (lung) cancer cells caused by galloylated catechins, such as (–)-gallocatechin gallate, (–)-EGCG and (–)-gallocatechin. The (–)-gallocatechin gallate 50 μmol L^{-1} inhibited proliferation of MCF-7, HCT-116 and NCI-H460 cells by 97, 93 and 67 %, respectively. EGCG 50 μmol L^{-1} was the most effective studied flavonoid, with 100 % inhibition of MCF-7 cells proliferation (25). Liang *et al.* (26) report-

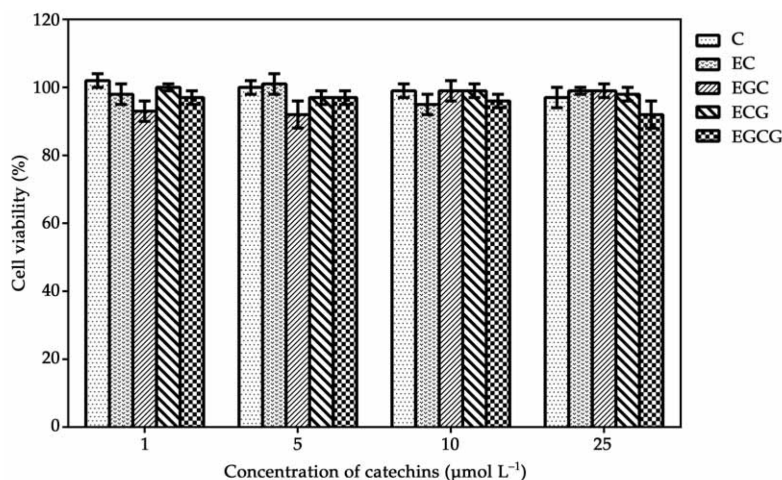

Fig. 1. The effect of catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin 3-gallate (ECG), and epigallocatechin gallate (EGCG) in concentrations of 1, 5, 10, and 25 µmol L^{-1} on the proliferation of cancer cells HCT-8. The exposition lasted 48 h. The data are expressed as percentage of the control cells number (cells exposed to vehicle 100 %) ± SD (n = 6).

ed the antiproliferative effect of epicatechin gallate (ECG) and EGCG in hepatocellular carcinoma cells BEL-7404, with the IC_{50} values of 1148 and 567 µmol L^{-1} for ECG and EGCG, respectively. This discrepancy could be explained by the differences in the cell line used and mainly by different concentrations. Much higher concentrations were used than in our experiments when the catechin concentrations ranged between 1 and 25 µmol L^{-1}, in the studies mentioned above. Systemic bioavailability of catechins after oral administration is low due to their poor absorption (27). For example, Chow *et al*. (28) reported maximal plasma concentration of EGCG of 1.7 µmol L^{-1}, after a single-dose oral administration of one tablet of Polyphenon E (a green tea extract containing 400 mg of EGCG) to healthy human volunteers. On the other hand, exposure of intestinal and colonic mucosa to these compounds is high (27).

Effect of catechins on antiproliferative activity of DOX in HCT-8 cells

DOX alone significantly inhibited cell proliferation in a concentration-dependent manner. The addition of catechins to DOX had no effect on cell viability. None of the studied catechins influenced the antiproliferative effect of DOX in the cancer cell line HCT-8 (Fig. 2).

Liang *et al*. (26) described the ability of ECG and EGCG to augment antitumor activity of DOX in the hepatocellular carcinoma cell line BEL-7404 as well as in murine xenografts. However, in these experiments very high concentrations of catechins (0.15–2.5 mmol L^{-1}), were used, which highly exceeded common plasmatic concentrations of these compounds. Stammler and Volm (29) showed a sensitizing effect of EGCG and EGC on the doxorubicin-resistant colon cancer cell line (SW620-dox) and murine sarcoma (S180-dox). In contrast, in our experiments, catechins in 5 µmol L^{-1} concentrations did not affect DOX

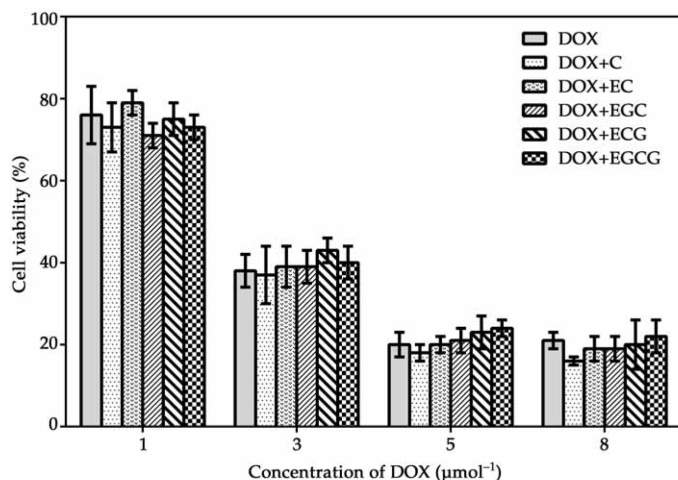

Fig. 2. The effect of catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin 3-gallate (ECG), and epigallocatechin gallate (EGCG) in concentration of 5 μmol L^{-1} on DOX concentration-dependent antiproliferative efficacy in cancer cells HCT-8. The exposition lasted 48 h. The data are expressed as percentage of the control cells number (cells exposed to vehicle 100 %) ± SD ($n = 6$).

antiproliferative efficacy in intestinal cancer cells. Addition of catechins caused no potentiation but also no decrease of DOX efficacy in these cancer cells. This finding indicates that concomitant consumption of low doses of catechins does not influence DOX therapy.

Effect of EGCG on the viability of isolated hepatocytes and on DOX-induced toxicity in hepatocytes

The potential health benefits associated with green tea consumption have been partially attributed to the antioxidative properties of polyphenols, particularly to catechins, among which EGCG is the most effective (30). Therefore, the effect of EGCG on DOX toxicity in non-cancerous cells was followed up. For this purpose, the primary culture of isolated hepatocytes was used. When the effect of EGCG (24-h exposure) on the viability of hepatocytes was tested, a mild increase ($p < 0.01$) in their viability was found only with the highest EGCG concentration (25 μmol L^{-1}),while lower concentrations of EGCG had no effect (Fig. 3). When hepatocytes were exposed for 24-h to DOX (10 and 25 μmol L^{-1}), a pronounced destruction of cells was observed. However, when hepatocytes were exposed to combinations of DOX (10 and 25 μmol L^{-1}) with 5 μmol L^{-1} EGCG, no damage occurred after 24-h exposure (Fig. 4). Protective effect of EGCG may depend on its ability to inhibit carbonyl reductase 1, the enzyme responsible for the formation of a more toxic DOX metabolite (21), and also on EGCG antioxidant and antiradical properties.

Hepatoprotective effect of catechin (200 mg per kg per week) in DOX-treated (5 mg per kg per week) rats was described by Kalendar *et al.* (5). Malondialdehyde level, glutathione peroxidase and catalase activities were significantly decreased in the catechin+ DOX-treated group compared to the DOX-treated group (5). These observations together with our results indicate that catechins could reduce DOX-induced hepatotoxicity.

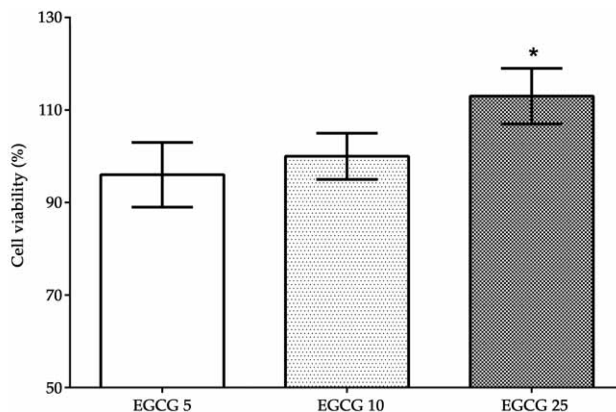

Fig. 3. The effect of epigallocatechin gallate (EGCG) at concentrations of 5, 10 and 25 μmol L^{-1} on the viability of isolated hepatocytes. The exposure lasted 24 h. The data are expressed as percentage of the control cells (cells exposed to vehicle 100 %) ± SD ($n = 6$). The viability of control cells represents 100 %. Asterisk indicates a significant difference from the control ($p < 0.01$).

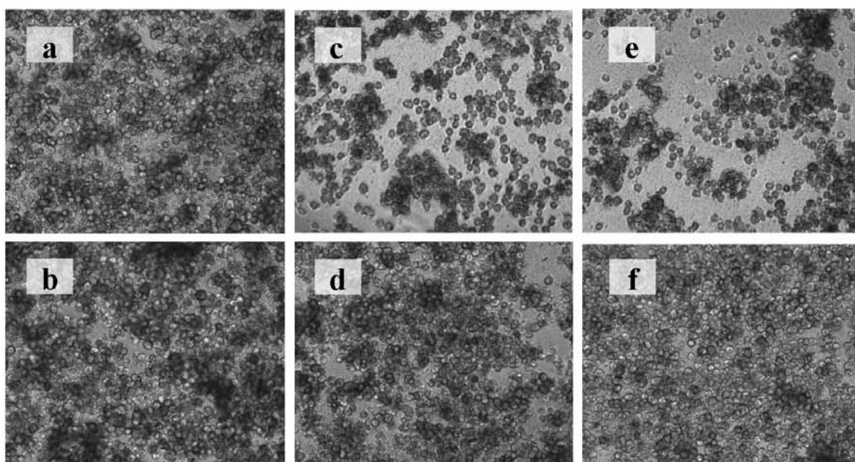

Fig. 4. Microscopic photo-documentation of the primary culture of isolated rat hepatocytes: a) without treatment, b) vehicle-treated, c) treated with 10 μmol L^{-1} DOX, d) treated with 10 μmol L^{-1} DOX + 5 μmol L^{-1} EGCG, e) treated with 25 μmol L^{-1} DOX, f) treated with 25 μmol L^{-1} DOX + 5 μmol L^{-1} EGCG, for 24 h.

Effect of EGCG on the DOX-induced formation of ROS in hepatocytes

To verify the antioxidant/antiradical mechanism of EGCG protective activity against DOX-mediated hepatotoxicity, the ROS-scavenging ability of EGCG in rat hepatocytes exposed to DOX was studied by the dichlorofluorescein assay. The results (Fig. 5) prov-

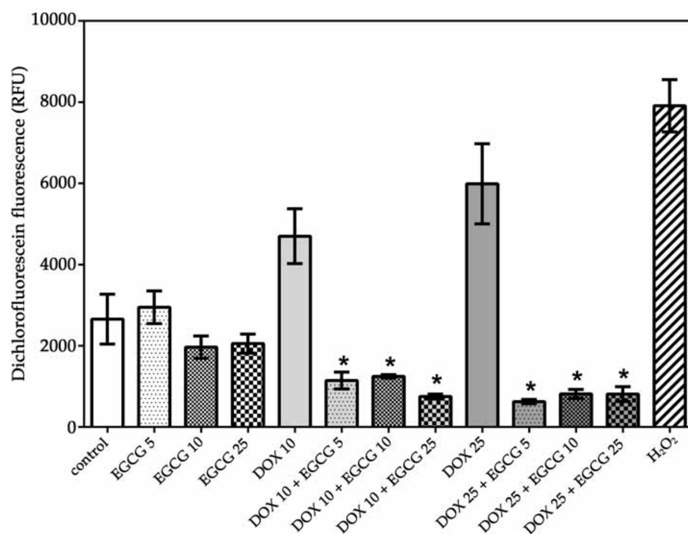

Fig. 5. Formation of ROS in hepatocytes exposed to epigallocatechin gallate (EGCG) in concentrations of 5, 10 and 25 µmol L^{-1}, to DOX in concentrations of 10 and 25 µmol L^{-1}, and to DOX + EGCG combinations. The exposition lasted 12 h. The 3 % H_2O_2 solution was used as a positive control. The data (relative fluorescence units) represent the mean ± SD (n = 6). Asterisk indicates a significant difference from DOX-treated cells (p < 0.01).

ed that DOX (10 and 25 µmol L^{-1}) markedly induced ROS formation in a concentration-dependent manner (p < 0.01). In control hepatocytes (without DOX), EGCG 25 µmol L^{-1} did not significantly decrease the basal formation of ROS, but it restricted ROS formation in hepatocytes exposed to DOX at 10 and 25 µmol L^{-1} by 84.0 and 86.4 % (p < 0.01), respectively. Even the lowest EGCG concentration (5 µmol L^{-1}) decreased the DOX-induced ROS formation up to 75.6 or 89.5 % in hepatocytes (DOX concentrations of 10 and 25 µmol L^{-1}, respectively).

Zheng *et al*. (18) reported protective action of EGCG against the DOX-induced ROS formation in cardiomyocytes. However, higher EGCG concentration (25 µmol L^{-1}) was necessary for a marked decrease of ROS formation in these cells (18).

CONCLUSIONS

Catechins had no influence on the proliferation of HCT-8 cancer cells and on the viability of isolated rat hepatocytes. Further, they did not interfere with the antiproliferative activity of DOX in the HCT-8 cancer cells. On the other hand, EGCG significantly protected rat hepatocytes against DOX-induced toxicity (at all tested concentrations), which was mainly caused by the formation of ROS. The protective effect of EGCG was probably caused by its antioxidant/antiradical properties. Taking together, consump-

tion of dietary supplements containing catechins, such as green tea, during chemotherapy with DOX, seems to be safe and beneficial, since catechins do not decrease anticancer efficacy of DOX but could attenuate DOX hepatotoxicity. However, the *in vivo* experiments (including animal models) should be performed for confirmation of the obtained results.

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