

ANTIOXIDATIVE EFFECT OF EPIGALLOCATECHIN GALLATE AGAINST D-GALACTOSAMINE-INDUCED INJURY IN PRIMARY CULTURE OF RAT HEPATOCYTES

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Summary: Literature data support that green tea and its major component epigallocatechin gallate (EGCG) have powerful antioxidant effects. Contrary, hepatotoxicity can be induced by high-dose EGCG. The timing of exposure to green tea in relation to administration of hepatotoxic agent plays an important role too. The aim of our work was a verification of antioxidant effect of EGCG on D-galactosamine-induced injury in primary culture of rat hepatocytes. Hepatocytes were incubated with EGCG at concentrations of 1.25–10 μ M and toxic D-galactosamine (GalN) for 24 hrs. Alternatively, hepatocytes were pretreated with EGCG for 24 hrs, and then incubated with EGCG and GalN for further 24 hrs. Cytotoxicity was analysed by lactate dehydrogenase activity, functional capacity by albumin production. Oxidative stress was evaluated from a production of malondialdehyde and glutathione content in the cells. EGCG protected hepatocytes against GalN-induced cytotoxicity but preventive treatment of intact hepatocytes with EGCG was required to diminish the development of hepatocyte injury. Oxidative stress induced in our study seems to overcome the ability of hepatocytes to improve GSH depletion and albumin production. Prolongation of the pretreatment with EGCG could be a promising strategy leading to amelioration of its hepatoprotective effect.

Key words: *Epigallocatechin gallate; Hepatocytes; D-galactosamine; Oxidative stress*

Introduction

Literature data indicate that green tea possesses a wide range of health-promoting effects including anti-cancer, anti-atherosclerotic, anti-diabetic, hypocholesterolemic or hypoglycemic activity (1–4). The main chemical components of unfermented green tea are polyphenols, the major ones are catechins, mainly epigallocatechin gallate (EGCG) which have powerful antioxidant effects. These compounds may be especially effective in preventing oxidative stress-related diseases (5). Liver injury is frequently associated with oxidative stress that plays a relevant role in the induction of cell death in hepatocytes. Therefore, antioxidative therapy represents a potential strategy to prevent liver injury. Experimental results support that oral treatment with green tea extracts can ameliorate a hepatotoxicity (6, 7). On the other side, there is a rising evidence that hepatotoxicity associated with increased lipid peroxidation can be induced by high-dose EGCG (8, 9). The timing of exposure to green tea in relation to administration of hepatotoxic agent plays an important role too (10).

D-galactosamine (GalN) is a highly selective hepatotoxin frequently used in experiments. GalN induces oxidative stress, apoptosis and necrosis in both liver tissue and cultured hepatocytes (11–13). *In vivo* GalN causes diffuse liver damage resembling viral hepatitis (14) thus contributing to the popularity of this experimental hepatotoxin.

Pharmacokinetic parameters of catechins are intensively studied. Intestine can play an important role in their low bioavailability how the catechins are conjugated here. Nevertheless, there are mostly the free catechins that seem to enter the tissues. *In vitro* model allow to eliminate the problem of low bioavailability and to identify easily the concentrations of the tested substance that are primarily nontoxic to hepatocytes. The aim of our work was a verification of potential antioxidant effect of the major catechin in green tea (EGCG) on GalN-induced injury in primary culture of rat hepatocytes. Moreover, two strategies were confronted: hepatocytes treated only simultaneously with EGCG and D-GalN or additionally pretreated with EGCG.

Materials and Methods

Chemicals

Medium William's E without phenol red, fetal bovine serum, penicillin, streptomycin and glutamine were purchased from Pan Biotech GmbH (Germany), D-galactosamine, type I collagen, trypan blue, epigallocatechin gallate from Sigma-Aldrich. Kit for lactate dehydrogenase (DiaSys, Germany), collagenase (Collagenase NB 4 Standard Grade from Clostridium histolyticum, Biotech, Czech Republic), insulin (Actrapid, Hoechst, Germany), glucagon (Glucagen, Novo Nordisk, Germany), prednisolon (Solu-Decortin, Merck,

USA), Rat Albumin Elisa Quantification Kit (Bethyl Lab. Inc., USA), and Cell Proliferation Reagent WST-1 (Roche, Germany) from suppliers mentioned in brackets.

Hepatocyte isolation, cultivation and treatment

All animal protocols were in full compliance with the guidelines for animal care approved by the Institutional Animal Use and Care Committee of the Charles University Prague, Czech Republic. Hepatocytes were isolated from male Wistar rats by collagenase perfusion (15). The viability of freshly isolated cells was more than 90% as confirmed by trypan blue exclusion test. Isolated hepatocytes were suspended in William's E medium supplemented with fetal bovine serum (6%), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (10 mg/ml), insulin (0.08 IU/ml), prednisolon (0.05 µg/ml), glucagon (0.008 µg/ml) and plated in collagen-coated 24-well plates at a density of 70,000/cm². Hepatocytes were allowed to attach in a gassed atmosphere (5% CO₂) at 37 °C for 2 h. After the establishment of monolayers, the medium was removed and replaced with a fresh supplemented medium without fetal bovine serum containing dissolved epigallocatechin gallate at different concentrations of 1.25, 2.5, 5 and 10 µM and toxic substance D-galactosamine (GalN, 40 mM). The treatment period lasted 24 hrs and then all medium was removed. Alternatively, hepatocytes were pretreated with EGCG for 24 hrs, and then the medium was replaced by medium with EGCG and toxic GalN for further 24 h. At the end of incubation period, the medium was collected for biochemical assays.

Biochemical assays

Hepatocyte toxicity was determined by the activity of lactate dehydrogenase (LDH) leakage. LDH activity in the culture medium and in lysate of hepatocytes (frozen and lysed cells in distilled water) was measured using a commercial kit. Concentration of malondialdehyde (MDA) was measured as TBARS in the culture medium (25) and served as a marker of lipid peroxidation. To evaluate the functional capacity of cultured hepatocytes the amount of albumin secreted into the culture medium during the incubation was measured using the ELISA kit. The nontoxic effect of EGCG on hepatocyte culture was tested by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. After cell lysis in a freezer (−80 °C, 10 min) and the harvesting of hepatocytes, glutathione content was determined by the spectrofluorometric assay (16).

Statistical analysis

Experiments were repeated at least three times using different isolations of hepatocytes. The results are expressed as a mean ±SD. Statistical significance was analysed using the one-way ANOVA test followed by Dunnett's post hoc

test (GraphPad Prism 6 for Windows, Graphpad Software, USA). $p < 0.05$ was considered to be statistically significant.

Results

Nontoxic effect of epigallocatechin gallate (EGCG) at the doses of 1.25, 2.5, 5 and 10 µmol/l was documented by WST-1 assay (Fig. 1). Concentrations from 20 µM were toxic.

The cytotoxicity induced in hepatocyte culture was investigated by measuring of LDH leakage (Fig. 2). D-galactosamine (GalN) caused significant increase in LDH leakage

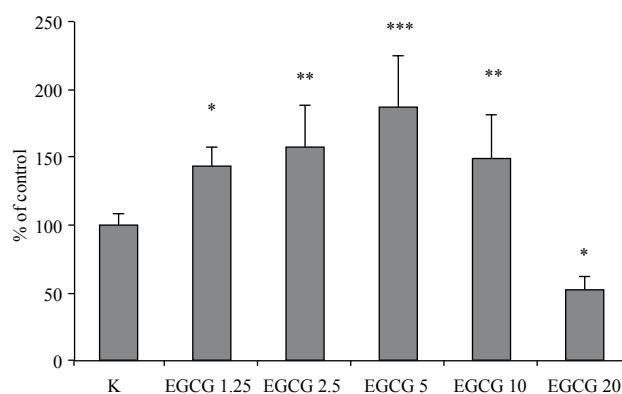


Fig. 1: Mitochondrial dehydrogenase activity of intact hepatocytes measured with WST-1 assay after 24 hours exposure to different concentrations of epigallocatechin gallate (EGCG) – 1.25; 2.5; 5; 10 and 20 µmol/l. WST of control hepatocytes is expressed as 100%. Each column represents the mean value and bars the S.D. (n = 8) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ difference from untreated control K.

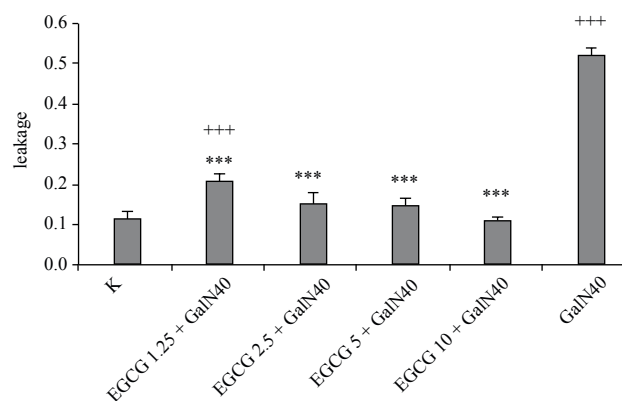


Fig. 2: Lactate dehydrogenase (LDH) leakage (the ratio of LDH activity in the medium and in the medium plus in the hepatocytes) after the pretreatment with epigallocatechin gallate (EGCG) – 1.25; 2.5; 5 and 10 µmol/l for 24 hours and further incubation with EGCG (1.25, 2.5, 5, 10 µM) and D-galactosamine (40 mM). Each column represents the mean value and bars the S.D. (n = 6). +++ $p < 0.001$ difference from K, *** $p < 0.001$ difference from GalN40. K: untreated control, GalN40: cultures treated with 40 mM D-galactosamine.

in comparison with control group ($p < 0.001$). This increase was prevented only when hepatocytes were preincubated with EGCG. EGCG significantly decreased LDH leakage at all concentrations we used ($p < 0.001$). However, simultaneous treatment of hepatocytes with EGCG and GalN did not protect LDH leakage. Therefore, LDH as well as markers of oxidative stress and functional parameters from the simultaneous treatment are not included in the results.

GalN induced a progressive increase in concentration of malondialdehyde (MDA, $p < 0.001$) which served as

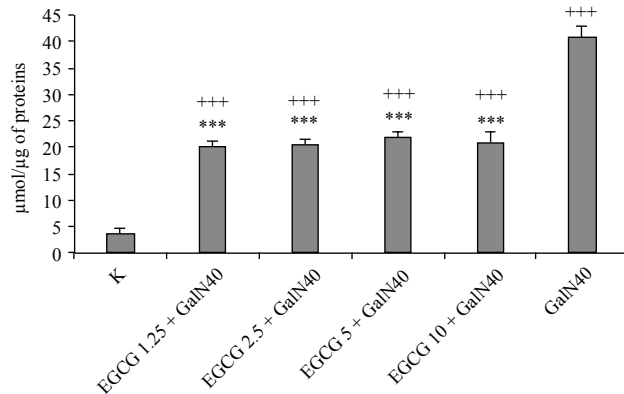


Fig. 3: Malondialdehyde production by hepatocytes after the pretreatment with epigallocatechin gallate (EGCG) – 1.25; 2.5; 5 and 10 $\mu\text{mol/l}$ for 24 hours and further incubation with EGCG (1.25, 2.5, 5, 10 μM) and D-galactosamine (40 mM). Each column represents the mean value and bars the S.D. ($n = 6$). +++ $p < 0.001$ difference from K, *** $p < 0.001$ difference from GalN40. K: untreated control, GalN40: cultures treated with 40 mM D-galactosamine.

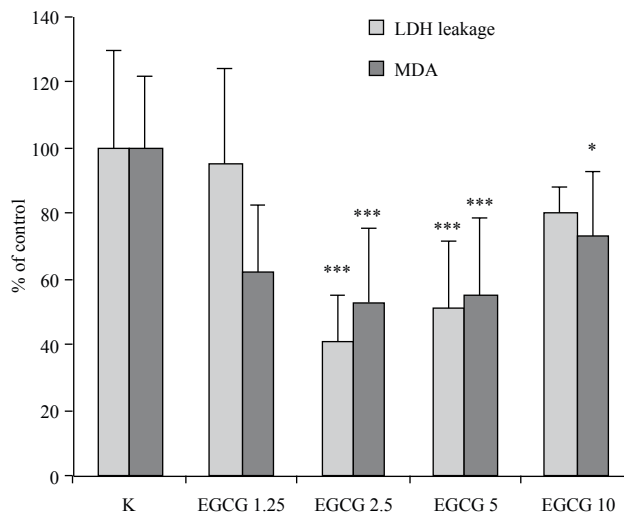


Fig. 4: Effect of epigallocatechin gallate (EGCG) – 1.25; 2.5; 5 and 10 $\mu\text{mol/l}$ on lactate dehydrogenase (LDH) leakage and malondialdehyde (MDA) production from intact hepatocytes. LDH leakage and MDA production of control hepatocytes is expressed as 100%. Each column represents the mean value and bars the S.D. ($n = 6$). * $p < 0.05$, *** $p < 0.001$ difference from untreated control K.

a marker of lipid peroxidation (Fig. 3). EGCG prevented GalN-induced lipid peroxidation in the same extent at all concentrations we used ($p < 0.001$). Fig. 4 documents that EGCG alone did not impair the integrity of hepatocyte cell membrane and did not increase the lipid peroxidation.

GalN alone caused a significant decrease in glutathione content (GSH+GSSG, $p < 0.001$). Glutathione content was not protected by the pretreatment with EGCG (Fig. 5). Never-

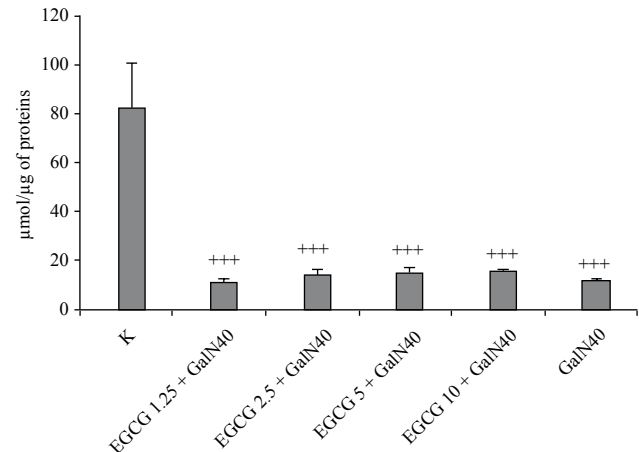


Fig. 5: Total content of glutathione (GSH+ GSSG) in hepatocytes after the pretreatment with epigallocatechin gallate (EGCG) – 1.25; 2.5; 5 and 10 $\mu\text{mol/l}$ for 24 hours and further incubation with EGCG (1.25, 2.5, 5, 10 μM) and D-galactosamine (40 mM). Each column represents the mean value and bars the S.D. ($n = 6$). +++ $p < 0.001$ difference from K. K: untreated control, GalN40: cultures treated with 40 mM D-galactosamine.

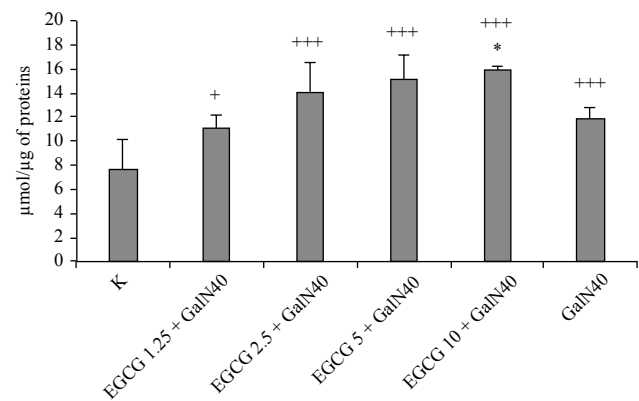


Fig. 6: Content of oxidized form of glutathione (GSSG) in hepatocytes after the pretreatment with epigallocatechin gallate (EGCG) – 1.25; 2.5; 5 and 10 $\mu\text{mol/l}$ for 24 hours and further incubation with EGCG (1.25, 2.5, 5, 10 μM) and D-galactosamine (40 mM). Each column represents the mean value and bars the S.D. ($n = 6$). + $p < 0.05$, +++ $p < 0.001$ difference from K, * $p < 0.05$ difference from GalN40. K: untreated control, GalN40: cultures treated with 40 mM D-galactosamine.

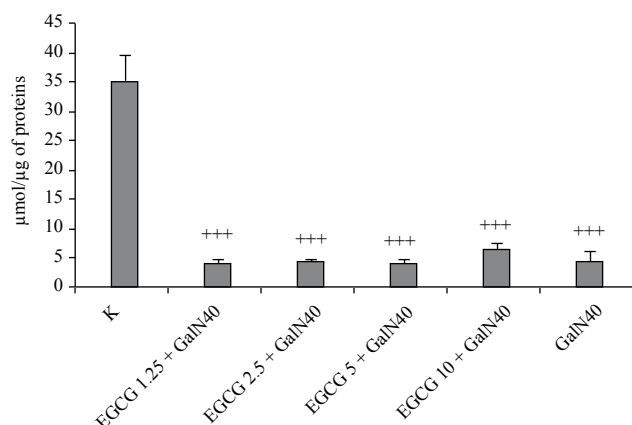


Fig. 7: Albumin production by hepatocytes after the pretreatment with epigallocatechin gallate (EGCG) – 1.25; 2.5; 5 and 10 $\mu\text{mol/l}$ for 24 hours and further incubation with EGCG (1.25, 2.5, 5, 10 μM) and D-galactosamine (40 mM). Each column represents the mean value and bars the S.D. ($n = 6$). +++ $p < 0.001$ difference from K. K: untreated control, GalN40: cultures treated with 40 mM D-galactosamine.

theless, GSSG significantly raised after the pretreatment with EGCG (Fig. 6) at the concentration of 10 $\mu\text{mol/l}$ ($p < 0.05$).

Concentration of albumin in culture medium served as a marker of functional capacity of hepatocytes. Albumin production by hepatocytes decreased after the incubation with GalN ($p < 0.001$). EGCG did not affect the functional capacity of hepatocytes at any concentration that we tested (Fig. 7).

Discussion

Flavonoids in green tea extracts have been found to play an important role in the protection against oxidative stress (17–19). As mentioned in Introduction antioxidative therapy represents a potential strategy to prevent liver injury. Literature data support the green tea and its major component EGCG as promising hepatoprotective agents. Nevertheless, an increasing evidence suggests that polyphenols can also behave as pro-oxidative agents leading to toxicity. Hepatotoxicity can be a consequence of high doses of green tea extracts (8, 9, 20) or by the time at which the treatment occurs. Green tea extract administered to mice prior to hepatotoxic dose of acetaminophen (APAP) attenuated the hepatotoxicity whereas; the opposite develops when green tea extract was administered after APAP (10). These effects have been mainly tested on animals after intragastric administration. There is a free EGCG that was determined in the tissues after both intragastric and intravenous applications. Passing the intestine, EGCG is largely conjugated in the plasma contrary to i.v. administration when tissues can be exposed to higher concentration of the free EGCG. The free EGCG seems to enter the tissues more readily. Moreover,

the lack of conjugated EGCG in the tissues may probably be due to its exclusion (21). Finally, the concentrations of EGCG in the tissues after intragastric treatment are lower than is expected from the plasma concentration. A relationship between dose and the percentage of conjugated versus unconjugated EGCG was not apparent (22). Therefore *in vitro* studies can better identify a dose of EGCG that promotes protective or toxic effect on hepatocytes. We have found that EGCG at concentrations of 1.25–10 μM are nontoxic to the intact rat hepatocytes in primary culture. Cytotoxic effect was exerted by concentrations from 20 μM . Cytotoxicity tested *in vitro* by other authors on hepalc1c mouse hepatocytes was pronounced dose dependently from 100 μM EGCG (8). Our work identifies that nontoxic 1.25–10 μM EGCG can prevent hepatocytes from D-galactosamine-induced cytotoxicity. An achievement of this protective effect depended on timing of EGCG treatment in relation to the administration of GalN. Preventive treatment of intact hepatocytes with EGCG was required to diminish the development of hepatocyte injury induced by GalN while only simultaneous treatment of hepatocytes with EGCG and GalN failed.

GalN-induced cell death is related to a rise of oxidative stress, increased production of reactive oxygen species (ROS) has been reported *in vivo* (23, 24) and *in vitro* (11, 13). In our experiment, the pretreatment of hepatocyte culture with EGCG protected a cell viability that paralleled an attenuation of lipid peroxidation induced by GalN. Catechins are effective scavengers. EGCG may decrease the concentration of lipid free radicals thus it may terminate the initiation and propagation of lipid peroxidation. When EGCG penetrates the lipid bilayer this protective effect can develop in the cytoplasmic membrane (25). Besides the induction of lipid peroxidation, GalN decreases GSH content in hepatocytes (12, 26). The cells respond to lipid peroxidation by antioxidant induction (27). GSH is an important protective molecule that acts as a free radical scavenger in the detoxification of reactive metabolites and ROS (10). There was documented that EGCG is preferentially used as an antioxidant, rather than GSH, to suppress lipid peroxidation and to protect cells from a decrease in GSH and oxidative damage (5, 28). In our study, while EGCG acts as an effective inhibitor of lipid peroxidation, decrease in total glutathione content (reduced, GSH, and oxidized, GSSG, forms) as well as its reduced form – GSH – was not protected. Nevertheless, we confirmed higher content of GSSG in hepatocytes pretreated with EGCG and further incubated with EGCG and hepatotoxic GalN. Green tea may increase GSH biosynthesis (29). We cannot exclude that intracellular GSH is more produced during our pretreatment of the intact hepatocytes with EGCG. Oxidative stress induced by GalN then can lead to oxidation of GSH in a higher extent in hepatocytes pretreated with EGCG. The oxidative stress may overcome the ability of the cells to reduce GSSG to GSH, leading to accumulation of GSSG within the cytosol (30). GSSG may also release the cell into medium (31) where the

concentration of GSH and GSSG were not measured in our work. Moreover, EGCG has been reported to react with GSH in isolated hepatocytes and to cause a transient decrease in GSH (10, 32). Then simultaneous cultivation with GalN (without preceding pretreatment with EGCG) could fail as the decrease in GSH stores deepens.

GalN also influences metabolic functions. In our conditions, GalN suppressed albumin synthesis. The toxicity of GalN is related to the depletion of uridine pools and alteration of RNA and protein synthesis (14). EGCG is not known to influence the content of uridine in hepatocytes. The oxidative stress induced by GalN is accompanied by activation of proinflammatory cytokines (23). These cytokines can cause dramatic alterations in hepatic gene expression further followed by decreased albumin synthesis (33–35). Therefore, we cannot exclude that in our study, the albumin production attenuated in response to the proinflammatory cytokines. However, literature data indicate protective effect of the green tea on release and action of proinflammatory cytokines (6, 23, 36) resulting in a reduction of hepatotoxicity, our finding did not confirmed the protective effect of EGCG on the albumin synthesis.

Conclusion

Only preventive treatment with EGCG protected rat hepatocytes from GalN induced toxicity in primary culture while EGCG given simultaneously with hepatotoxin failed. The reduction of lipid peroxidation can contribute to the protection against disruption of the cellular membrane. In spite of the effect of EGCG on GSH production cannot be excluded, the oxidative stress induced in our study seems to overcome the ability of hepatocytes to improve GSH depletion. This can result to the finding that the functional capacity of hepatocytes was not protected satisfactory. Prolongation of the pretreatment with EGCG could be a promising strategy leading to amelioration of its hepatoprotective effect.

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