

# Epigallocatechin Gallate Does Not Accelerate the Early Phase of Liver Regeneration After Partial Hepatectomy in Rats

Vojtěch Mezera · Otto Kučera · Alena Moravcová ·  
Eva Peterová · Zuzana Červinková

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## Abstract

**Background** Two-thirds partial hepatectomy (PHx) is an established model for the study of liver regeneration after resection. This process is accompanied by oxidative stress.

**Aims** In our study, we tested the effect of epigallocatechin gallate (EGCG), a green tea antioxidant, on the early phase of liver regeneration after PHx.

**Methods** Male Wistar rats were divided into five groups: (I) laparotomy + water for intraperitoneal injections, (II) laparotomy + EGCG 50 mg/kg body weight, (III) PHx + water for injections, (IV) PHx + EGCG 20 mg/kg and (V) PHx + EGCG 50 mg/kg, for 3 consecutive days. The rats were killed 24 h after surgery. Biochemical analysis of rat sera was performed. Histological samples were stained with hematoxylin & eosin and bromodeoxyuridine (BrdU). In hepatectomized rats, we also measured plasma malondialdehyde, tissue malondialdehyde, glutathione and cytokines levels, the activity of caspases 3/7, expression of

Nqo-1 and HO-1 genes at the mRNA level, and expression of p21, p-p27 and p-p53 genes at the protein level.

**Results** We observed lower accumulation of BrdU in group V when compared to groups III and IV. The activity of caspases 3/7 and expression of p-p53 were lower in group V than in groups III and IV. Tissue levels of IL-6 were lower in group V when compared to group III. Significant differences were not noted in other parameters.

**Conclusions** Administration of EGCG did not stimulate early phase liver regeneration in rats after PHx. There was even lower DNA synthesis in the group treated with a high dose of EGCG.

**Keywords** Antioxidants · Epigallocatechin gallate · Hepatectomy · Liver regeneration · Oxidative stress

## Abbreviations

|        |   |
|--------|---|
| AI     | Water for injections                          |
| ALT    | Alanine aminotransferase                      |
| ALP    | Alkaline phosphatase                          |
| AST    | Aspartate aminotransferase                    |
| BrdU   | 5-Bromo-2-deoxyuridine                        |
| EGCG   | Epigallocatechin gallate                      |
| EGCG20 | Epigallocatechin gallate 20 mg/kg body weight |
| EGCG50 | Epigallocatechin gallate 50 mg/kg body weight |
| GSH    | Glutathione (reduced form)                    |
| GSSG   | Glutathione (oxidized form)                   |
| HO-1   | Heme oxygenase-1                              |
| HRR    | Hepatic regeneration rate [25]                |
| IL-6   | Interleukin-6                                 |
| LAP    | Laparotomy                                    |
| LRR    | Liver regeneration rate [26]                  |
| MDA    | Malondialdehyde                               |

V. Mezera (✉) · O. Kučera · A. Moravcová · Z. Červinková  
Department of Physiology, Faculty of Medicine in Hradec  
Kralove, Charles University in Prague, Simkova 870,  
500 38 Hradec Kralove 1, Czech Republic  
e-mail: mezev5ar@lfhk.cuni.cz

O. Kučera  
e-mail: kucerao@lfhk.cuni.cz

A. Moravcová  
e-mail: moravca2@lfhk.cuni.cz

Z. Červinková  
e-mail: wolff@lfhk.cuni.cz

E. Peterová  
Department of Biochemistry, Faculty of Medicine in Hradec  
Kralove, Charles University in Prague, Hradec Kralove 1, Czech  
Republic  
e-mail: peterovae@lfhk.cuni.cz

|                |                                  |
|----------------|----------------------------------|
| Nqo-1          | NAD(P)H-quinone-oxidoreductase   |
| Nrf2-2         | NF-E2-related factor 2           |
| PHx            | Two-thirds partial hepatectomy   |
| TGF- $\beta$ 1 | Transforming growth factor beta1 |
| TNF- $\alpha$  | Tumor necrosis factor alpha      |

## Introduction

Liver resection is a surgical procedure performed for a variety of malignant and benign focal liver lesions. It is one of the few methods that gives the patient a chance for complete recovery. This is in both primary liver tumors, especially hepatocellular carcinoma [1], and secondary tumors, notably liver metastases from colorectal carcinoma [2].

Vascular occlusion, used during liver resection to prevent hemorrhage, causes ischemia–reperfusion injury. Reperfusion of the liver enhances cell injury by oxidative stress and an inflammatory reaction [3]. In addition, liver regeneration itself was found to be a state of increased oxidative stress [4, 5]. Some surgical procedures for preventing the ischemia–reperfusion injury have already found their way into clinical practice [3], whereas possible pharmacological approaches remain limited. This is because no decrease in mortality, incidence of liver failure or perioperative morbidity was found [6]. Two-thirds partial hepatectomy (PHx), carried out on small laboratory animals, is a well-established model for studying of liver regeneration [7].

Excessive oxidative stress observed in mice after knockout of a key transcription factor for endogenous antioxidative enzymes led to significant diminution of liver regeneration after PHx [8]. This transcription factor, NF-E2-related factor 2 (Nrf2), is an upstream regulator of various antioxidative proteins and xenobiotic-metabolizing enzymes, particularly glutathione-*S*-transferase, NAD(P)H-quinone-oxidoreductase (Nqo-1), superoxide-dismutase, glutathione-peroxidase, heme oxygenase-1 (HO-1), catalase and others [8–10].

Protection against oxidative stress may enhance liver regeneration after resection, as shown in a study using vitamins C and E [11]. Aside from these directly acting antioxidants, which after oxidation can act as pro-oxidants, indirect antioxidants exist. These are able to activate the aforementioned transcription factor Nrf2 and subsequently many cytoprotective proteins [9, 10]. The indirectly acting antioxidants have very low prooxidative effects [12].

One of these is (–)-epigallocatechin-3-gallate (EGCG), a polyphenolic antioxidant from green tea. Besides this effect, EGCG also acts as a direct antioxidant because of

the presence of phenolic groups that are sensitive to oxidation and can generate quinone [9, 13]. EGCG is the most abundant of the green tea catechins [14]. Its protective role was found in a model of acute toxic liver injury [15], chronic toxic liver injury [16, 17], non-alcoholic steatosis and steatohepatitis [18–20], and hepatic ischemia–reperfusion injury [18, 21]. EGCG was also able to ameliorate experimental diabetes mellitus by downregulation of genes for gluconeogenesis and synthesis of fatty acids, triacylglycerols and cholesterol [22, 23]. The effect of EGCG on liver regeneration after PHx has not been tested yet.

In our study, we tested the effect of EGCG in the early phase of liver regeneration after PHx in rats. Since the highest rate of DNA synthesis is in hepatocytes 24 h after PHx [24], we decided to investigate the regenerative response in this particular interval. We also focused on the monitoring of possible changes in relevant signaling pathways.

## Methods

### Chemicals

Unless otherwise stated, BrdU (5-bromo-2-deoxyuridine) and other chemicals used were all of analytical grade and had been purchased from Sigma-Aldrich (St. Louis, MO, USA). The kit for determination of caspase 3/7 activities was obtained from Promega (Madison, WI, USA). Mouse monoclonal anti-BrdU antibody had been obtained from DAKO (Glostrup, Denmark) and secondary anti-mouse antibody from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Mouse anti- $\beta$ -actin antibodies and rabbit anti-p21 CIP1 were purchased from Sigma-Aldrich, rabbit p27 Kip1 and rabbit phospho-p53 (Ser15) from Cell Signaling (Beverly, MA, USA), mouse Bcl-2 antibody (C-2) from Santa Cruz Biotechnology (Dallas, TX, USA), secondary goat anti-rabbit IgG from Sigma-Aldrich and secondary goat anti-mouse IgG from Santa Cruz Biotechnology.

### Experimental Design

Male Wistar rats (BioTest, Konárovice, Czech Republic) with initial weight of  $248 \pm 13$  g were used throughout the study. The rats were housed at  $23 \pm 1$  °C,  $55 \pm 10$  % humidity, air exchange 12–14 times/h and 12 h light–dark cycles. The animals had free access to tap water and were fed the standard pelleted diet ST-1 ad libitum (Velas, Lysá nad Labem, Czech Republic). In accordance with Czech legislation, all animals received care according to the guidelines set out by the Institutional Animal Use and Care

Committee of the Faculty of Medicine of Hradec Králové, and this committee approved our study.

The animals were selected for laparotomy (LAP, sham operation) or 2/3 partial hepatectomy (PHx) under ether anesthesia [7]. Water for injections (AI) or EGCG in a dose of 20 or 50 mg/kg body weight was administered intraperitoneally for 3 consecutive days (regimen according to [15]), with the third dose administered immediately after the operation. The animals were divided into following five groups:

I—AI + LAP: water for injections and laparotomy,  $n = 6$ ;

II—EGCG50 + LAP: EGCG 50 mg/kg and laparotomy,  $n = 6$ ;

III—AI + PHx: water for injections and PHx,  $n = 6$ ;

IV—EGCG20 + PHx: EGCG 20 mg/kg and PHx,  $n = 6$ ;

V—EGCG50 + PHx: EGCG 50 mg/kg and PHx,  $n = 6$ .

The animals were killed under general anesthesia by exsanguination 24 h after the operation, i.e., on the 4th day of the experiment. BrdU for detection of DNA synthesis was administered at a dose of 50 mg/kg intraperitoneally 1 h before sacrificing. Blood and liver samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. Resected liver from the 3rd day and the remnant on the 4th day were weighed and liver growth was calculated according to formulas from the literature [25, 26].

### Serum Biochemical Measurements

Serum concentrations of glucose, total and conjugated bilirubin, activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) of all animals were measured in the Department of Clinical Biochemistry and Diagnostics, University Hospital in Hradec Králové. In the hepatectomized groups (groups III, IV, V), plasmatic levels of malondialdehyde (MDA) were also measured.

### Determination of GSH and GSSG

After adding the liver homogenate to cold 10 % metaphosphoric acid, mixing and centrifuging ( $20,000\times g$ , 10 min.,  $4^{\circ}\text{C}$ ), glutathione in the supernatant was analyzed by Hissin's fluorimetric method [27] modified by Roušar [28]. Briefly, GSH was exposed to orthophthalaldehyde in hydrogenphosphate buffer, and the fluorimetric detection was carried out ( $\lambda Ex = 340\text{ nm}$ ,  $\lambda Em = 420\text{ nm}$ ). GSSG was assessed after alkaline hydrolysis in the same way as GSH.

### Determination of Liver Tissue Cytokines and TBARS

The protein content in the sample was determined after homogenization in RIPA buffer and spinning ( $10,000\times g$ ) by the Bradford method [29] using bovine serum albumin as a standard. Afterwards, levels of liver TNF- $\alpha$  in the supernatant were measured using the ELISA method. Rat TNF-alpha Platinum ELISA, Rat IL-1 beta Platinum ELISA and Rat TGF-beta1 Platinum ELISA kits were purchased from eBioscience (San Diego, CA, USA).

Lipid peroxidation in liver supernatants from the homogenates was determined according to Ohkawa et al. [30], i.e., by the assessment of thio barbituric acid reactive substances (TBARS).

### Determination of Caspase-3/7 Activity in Liver Homogenate

Activity of executive caspases was measured using the Caspase-Glo<sup>TM</sup> 3/7 kit. Briefly, after homogenization according to Liu et al. [31], the tissues were centrifuged and samples adjusted to contain the same amount of protein [29]. The samples were incubated for 1 and 2 h with Caspase-Glo<sup>TM</sup> reagent (contains substrate for caspases 3/7), and the emitted luminescence was measured using the TECAN Infinite M200.

### Determination of p21, p-p27, p-p53, and Bcl-2 Expression

Expression of p21, p-p27 and p-p53 genes at the protein level was estimated using the Western blot method as described previously [32]. After homogenization [31] and quantification of protein content [29], the lysates containing an equal amount of protein (10  $\mu\text{g}$ ) were subjected to SDS-PAGE using gradient gel NuPAGE<sup>®</sup> Novex<sup>®</sup> Bis-Tris Mini gels 4–12 % (Life Technologies, Carlsbad, CA, USA) and transferred to a cellulose nitrate membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with primary rabbit antibodies at  $4^{\circ}\text{C}$  overnight and with secondary antibody goat anti-rabbit IgG for 2 h. Proteins were visualized using a chemiluminescence detection kit (Roche, Penzberg, Germany) and autoradiographic film (Foma, Hradec Kralove, Czech Republic). To confirm equal protein loading, each membrane was reprobated and reincubated to detect  $\beta$ -actin. The preparation differed for Bcl-2 protein, where we used a more concentrated homogenate of liver tissue in RIPA buffer.

### Determination of HO-1 and Nqo-1 Gene Expression

Gene expression was examined as previously described [33], with the exception that total RNA from liver tissue was isolated by phenol–chloroform extraction using the

RNA Blue kit (TOP-Bio, Prague, Czech Republic). TaqMan Fast Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assay kits were from Life Technologies (Carlsbad, CA, USA), and primers and probe were purchased from Generi Biotech (Hradec Králové, Czech Republic). Glyceraldehyde-3-phosphate dehydrogenase was used as reference for normalizing the data.

Liver Histology, Incorporation of Bromodeoxyuridine

After the rats had been killed, liver samples were immediately fixed by immersion in 4 % neutral formaldehyde. Paraffin sections were stained with hematoxylin & eosin and checked for the presence of steatosis and/or inflammation. Furthermore, apoptosis was assessed according to [34], i.e., the number of apoptotic bodies per visual field was calculated in hematoxylin & eosin-stained samples. The pathologist evaluating the samples was blinded to the study.

The immunohistochemical analysis was made on paraffin sections of the liver tissue as described previously [35]. Briefly, the paraffin sections were incubated with primary mouse anti-BrdU monoclonal antibody, then with biotinylated anti-mouse secondary antibody and subsequently with a streptavidin conjugate of peroxidase (Dako, Glostrup, Denmark). Visualization of bound antibody was performed using 3,3'-diaminobenzidine-tetrahydrochloride and hydrogen peroxide. BrdU incorporation was determined by counting of positively stained hepatocyte nuclei in nine representative microscope fields (×100 magnification) using an Olympus IX51 microscope and quantified using a computer-aided image analysis system NIS-Elements AR 2.30 (Nikon, Lewisville, TX, USA).

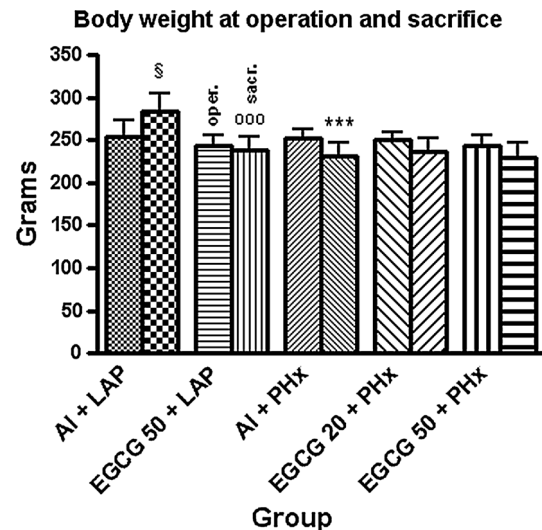
Statistical Analysis

The results are expressed as the mean ± SD. Analyses were performed using Graph-Pad Prism 4.03 software (Graph Pad Software, San Diego, CA, USA). First, normality was tested by Kolmogorov-Smirnov test. In normal data, comparisons were made among the groups using ANOVA followed by Tukey-Kramer's post hoc test. In the case of non-Gaussian distribution, non-parametric Kruskal-Wallis tests and Dunn's post hoc test were used.  $p < 0.05$  was considered statistically significant.

Results

Animals, Body and Liver Weight

During our experiment all animals survived until the sacrifice. One animal from the EGCG50 + PHx group had a surgical complication (hemoperitoneum) and was excluded



**Fig. 1** Body weight of experimental animals at operation (day 1) and at sacrifice (day 2). §, §§, §§§§When comparing the same group at sacrifice and at operation. °, °°, °°°When comparing the EGCG50 + LAP group with the AI + LAP group; \*, \*\*, \*\*\* when compared to laparotomized animals (AI + PHx vs. AI + LAP; EGCG20 + PHx vs. EGCG50 + LAP; EGCG50 + PHx vs. EGCG50 + LAP),  $p < 0.05$ ;  $p < 0.01$ ;  $p < 0.001$ , respectively

**Table 1** Indices of liver regeneration

|         | AI + PHx | EGCG 20 + PHx | EGCG 50 + PHx | p value |
|---------|----------|---------------|---------------|---------|
| HRR (%) | 9.5 ± 4  | 13.5 ± 6      | 8.9 ± 10      | ns      |
| LRR (%) | 23.3 ± 8 | 29.9 ± 9      | 18.1 ± 25     | ns      |

$HRR (\%) = (C - (A - B)) / A \times 100$  (Murata [26])

$LRR (\%) = (C - (A - B)) / C \times 100$  (Selzner [25])

A estimated total liver weight at PHx, B excised liver weight, C weight of regenerated liver at sacrifice

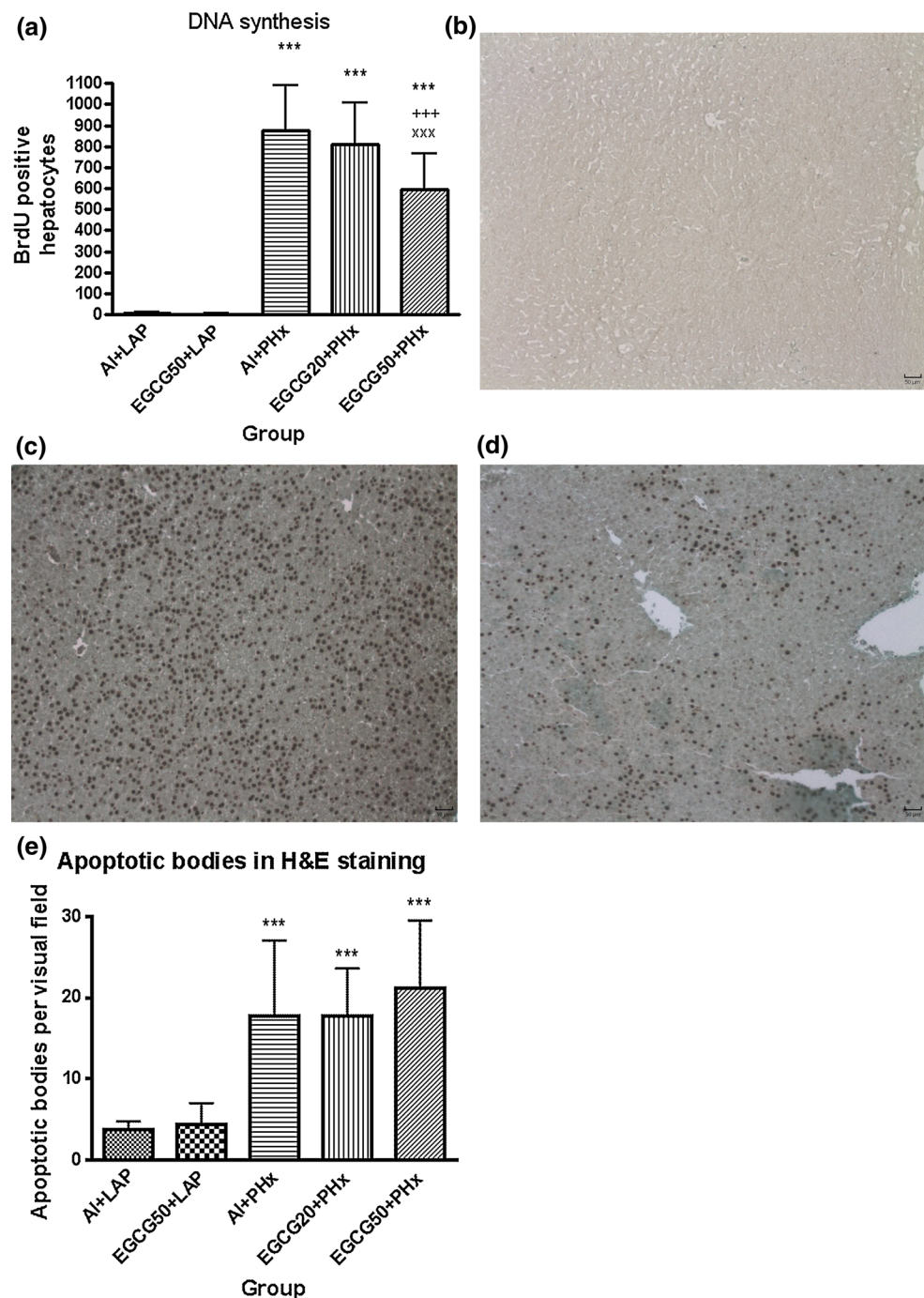
from further analysis. In group I (AI + LAP) the body weight rose after the operation ( $p < 0.05$ ); in all other groups, there was a non-significant trend to decline in body weight after the operation. Body weight at operation did not differ among groups; however, body weight was higher at sacrifice in the AI + LAP group when compared to the EGCG50 + LAP and AI + PHx groups ( $p < 0.001$ ) (Fig. 1).

Among hepatectomized groups (III, IV, V), the calculated indices of liver regeneration appeared highest in the EGCG20 + PHx group; however, due to high variability the differences were not statistically significant (Table 1).

Histological Findings, Incorporation of BrdU

In hematoxylin & eosin staining from the caudate lobe, distinct lipid accumulation was visible in the hepatectomized groups (III, IV, V); this is consistent with published

**Fig. 2** **a** DNA synthesis estimated by BrdU incorporation. \*, \*\*, \*\*\* When compared to laparotomized animals (AI + PHx vs. AI + LAP; EGCG20 + PHx vs. EGCG50 + LAP; EGCG50 + PHx vs. EGCG50 + LAP); +, ++, +++ when compared to the AI + PHx group; ×, ××, ××× when compared to the EGCG20 + PHx group,  $p < 0.05$ ;  $p < 0.01$ ;  $p < 0.001$ , respectively. **b** Laparotomized groups: only sporadic BrdU-positive cells. **c** Group III (AI + PHx): numerous BrdU-positive cells. **d** Group V (EGCG50 + PHx): numerous BrdU-positive cells, but less than in groups III and IV. Note that the positive nuclei are mostly in periportal areas. **e** Apoptotic bodies. \*, \*\*, \*\*\* When compared to laparotomized animals (AI + PHx vs. AI + LAP; EGCG20 + PHx vs. EGCG50 + LAP; EGCG50 + PHx vs. EGCG50 + LAP)



data [36]. In addition, there was vacuolation of the cytoplasm in peripherally located hepatocytes in the hepatectomized groups and sporadically occurring pyknotic nuclei. In the center of the lobe, sparse hepatocytes with granular eosinophilic cytoplasm occurred. We did not observe signs of pronounced inflammatory infiltration in any of the rats. The number of apoptotic bodies was significantly higher in the hepatectomized groups (III, IV, V) compared to the laparotomized groups (I, II). The apoptotic bodies were mostly noticed around the central veins. The laparotomized

groups did not differ from one another in apoptotic body count; we did not observe differences among the hepatectomized animals either (Fig. 2e).

Immunohistochemical staining for incorporated BrdU showed only sporadic positive hepatocytes in the laparotomized groups (I, II); there was high positivity in the hepatectomized groups (III, IV, V). We observed no difference between groups III and IV; however, the count of BrdU-positive cells in the EGCG50 + PHx group was lower when compared to the AI + PHx and

**Table 2** Serum biochemistry

|                          | AI + LAP    | EGCG 50 + LAP | AI + PHx       | EGCG 20 + PHx | EGCG 50 + PHx  | <i>p</i> value |
|--------------------------|-------------|---------------|----------------|---------------|----------------|----------------|
| Glycemia (mmol/l)        | 7.01 ± 0.61 | 10.49 ± 0.28° | 7.97 ± 0.57    | 8.9 ± 0.69*   | 8.71 ± 0.75*   | <0.001         |
| ALT (μkat/l)             | 1.01 ± 0.57 | 0.64 ± 0.06   | 4.08 ± 4.76    | 2.7 ± 1.38**  | 5.44 ± 4.95**  | <0.01          |
| AST (μkat/l)             | 3.2 ± 1.99  | 1.89 ± 0.37   | 7.05 ± 6.34    | 6.33 ± 2.57** | 9.92 ± 8.20**  | <0.01          |
| ALP (μkat/l)             | 2.4 ± 0.45  | 2.57 ± 0.32   | 7.08 ± 1.11*** | 6.8 ± 1.29*** | 6.61 ± 1.39*** | <0.001         |
| Bilirubin total (μmol/l) | 2.0 ± 0.0   | 3.0 ± 0.0     | 4.0 ± 1.55*    | 3.5 ± 0.55    | 5.6 ± 2.19     | <0.05          |
| Bilirubin conj. (μmol/l) | 1.83 ± 0.41 | 2.0 ± 0.0     | 2.6 ± 0.89     | 2.17 ± 0.41   | 4.2 ± 2.28*    | <0.05          |

°, °°, °°°When comparing the EGCG50 + LAP with the AI + LAP group

\*, \*\*, \*\*\* When compared to laparotomized animals (AI + PHx vs. AI + LAP; EGCG20 + PHx vs. EGCG50 + LAP; EGCG50 + PHx vs. EGCG50 + LAP), *p* < 0.05; *p* < 0.01; *p* < 0.001, respectively

EGCG20 + PHx groups (*p* < 0.001 for both comparisons) (Fig. 2a–d). BrdU positivity and thus DNA synthesis were noticed mostly in the periportal areas (Fig. 2c–d).

### Serum Biochemical Characteristics

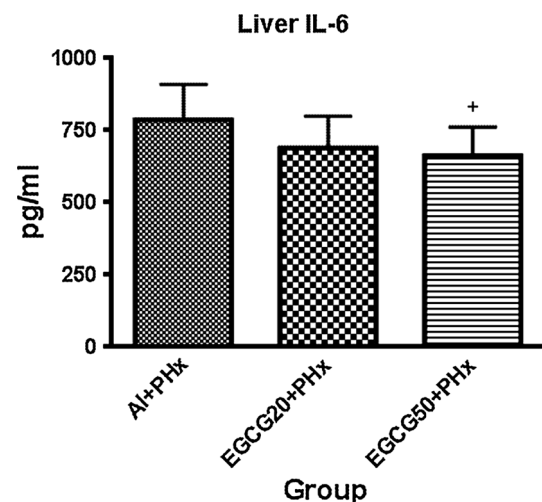
Serum glycemia was higher in group II (EGCG50 + LAP) when compared to group I (AI + LAP). There was a trend towards hyperglycemia even at a dose of 20 mg/kg when comparing group IV (EGCG20 + PHx) with group III (AI + PHx). In addition, we observed lower glycemia in the hepatectomized groups treated with EGCG when compared to the EGCG50 + LAP group.

Alanine aminotransferase and aspartate aminotransferase activity was higher in hepatectomized animals, but the statistical significance was present only when comparing EGCG20 + PHx and EGCG50 + PHx with EGCG50 + LAP. Furthermore, the ALT and AST activity was somewhat lower in the EGCG20 + PHx than in the AI + PHx group, but this difference did not reach statistical significance. Alkaline phosphatase activity was higher in all hepatectomized groups when compared to the laparotomized groups.

Total bilirubin was higher in the EGCG50 + LAP group than in the AI + LAP group, and in the hepatectomized than in the laparotomized groups; however, a significant difference was observed only when comparing the AI + PHx with the AI + LAP groups. Conjugated bilirubin was higher in the hepatectomized than in the laparotomized groups, reaching significance only when comparing the EGCG50 + PHx with the EGCG50 + LAP group. Both total and conjugated bilirubin reached their highest values in the EGCG50 + PHx group; however, the differences among hepatectomized groups were not statistically significant (Table 2).

### Tissue Cytokines TNF-α, IL-6, and TGF-β: Markers of Oxidative Stress

We observed an insignificant trend of lower TNF-α in hepatectomized groups treated with EGCG when compared



**Fig. 3** Tissue levels of IL-6. +, ++, +++When compared to the AI + PHx group, *p* < 0.05; *p* < 0.01; *p* < 0.001, respectively

to the AI + PHx group. A similar trend was observed with IL-6, and its expression was significantly lower in the EGCG50 + PHx group when compared to the AI + PHx group (Fig. 3). We did not observe differences in the expression of liver TGF-β1.

A similar non-significant trend of lower tissue MDA, GSH and GSSG content as well as lower plasmatic MDA was observed in the EGCG20 + PHx group when compared to the AI + PHx and EGCG50 + PHx groups; however, none of these reached the level of statistical significance (Table 3).

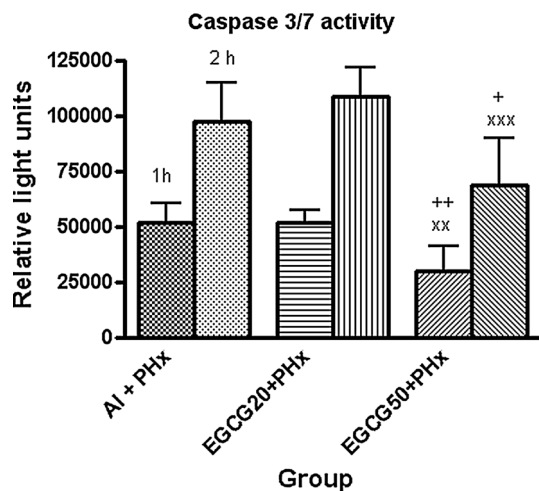
### Nqo-1 and HO-1 Gene Expression at the mRNA Level

There were no significant differences among hepatectomized groups in mRNA expression of Nqo-1 and HO-1. Because of the small amount of measured samples in experimental groups (*n* = 3), we observed only an insignificant trend of lower HO-1 expression in EGCG20 + PHx and higher HO-1 expression in the

**Table 3** Cytokines TNF- $\alpha$ , IL-6 and TGF- $\beta$ : markers of oxidative stress

|                                     | AI + PHx          | EGCG 20 + PHx     | EGCG 50 + PHx             | <i>p</i> value |
|-------------------------------------|-------------------|-------------------|---------------------------|----------------|
| Plasmatic MDA ( $\mu\text{mol/l}$ ) | 1.20 $\pm$ 0.18   | 1.07 $\pm$ 0.18   | 1.24 $\pm$ 0.14           | ns             |
| Tissue MDA (nmol/mg protein)        | 0.35 $\pm$ 0.20   | 0.27 $\pm$ 0.07   | 0.47 $\pm$ 0.13           | ns             |
| Tissue GSH ( $\mu\text{mol/l}$ )    | 810 $\pm$ 195     | 717 $\pm$ 164     | 781 $\pm$ 144             | ns             |
| Tissue GSSG ( $\mu\text{mol/l}$ )   | 238 $\pm$ 169     | 188 $\pm$ 140     | 241 $\pm$ 164             | ns             |
| Tissue TNF- $\alpha$ (pg/ml)        | 803 $\pm$ 103     | 771 $\pm$ 69      | 752 $\pm$ 126             | ns             |
| Tissue IL-6 (pg/ml)                 | 785 $\pm$ 122     | 686 $\pm$ 111     | 660 $\pm$ 99 <sup>+</sup> | <0.05          |
| Tissue TGF- $\beta$ 1 (pg/ml)       | 8,024 $\pm$ 4,775 | 6,340 $\pm$ 4,053 | 7,647 $\pm$ 4,651         | ns             |

+, ++, +++ When comparing the EGCG50 + PHx with the AI + PHx group,  $p < 0.05$ ;  $p < 0.01$ ;  $p < 0.001$ , respectively



**Fig. 4** Activity of executive caspases 3/7; incubation for 1 and 2 h, respectively. +, ++, +++When compared to the AI + PHx group; ×, ××, ×××when compared to the EGCG20 + PHx group,  $p < 0.05$ ;  $p < 0.01$ ;  $p < 0.001$ , respectively

EGCG50 + PHx group when compared to the AI + PHx group (not shown).

#### Activity of Caspases 3/7

After 1 h incubation, lower activity in the EGCG50 + PHx group was observed when compared to the AI + PHx and EGCG20 + PHx groups ( $p < 0.01$  for both comparisons). After 2 h incubation, the EGCG50 + PHx group also had lower activity compared to both the AI + PHx ( $p < 0.05$ ) and EGCG20 + PHx ( $p < 0.001$ ) groups (Fig. 4).

#### Protein Expression of Cell-Cycle Regulating Proteins

Protein expression of p21 and p-p27 did not differ among hepatectomized groups. On the other hand, expression of p-p53 protein appeared higher in group IV (EGCG20 + PHx) and lower in group V (EGCG50 + PHx) than in group III (AI + PHx) (Fig. 5). We were not able to detect Bcl-2 protein.

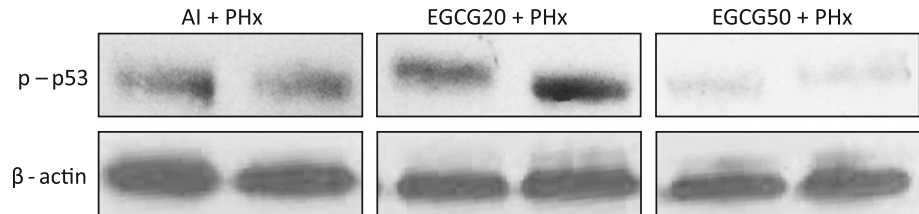
#### Discussion

Despite several studies describing the beneficial effect of EGCG on the liver in various experimental models, there was no beneficial effect on liver regeneration after PHx in our experiment. There was even lower DNA synthesis in animals receiving higher doses of EGCG. Based on our results, the use of EGCG in conditions of organ alteration, such as regenerating liver, cannot be recommended. The effect of EGCG is complex: several hundred EGCG-regulated genes were identified in a study with microarrays [37]. Furthermore, anti-proliferative and -cancer properties of EGCG are known [38, 39].

Since EGCG was reported to decrease the production of TNF- $\alpha$  [40] and IL-6 [38], which plays an important role in liver regeneration [41, 42], we wondered if the inhibiting effect of EGCG on DNA synthesis was mediated in this way. There was only a non-significant trend of lower TNF- $\alpha$  levels in EGCG-treated hepatectomized animals when compared to hepatectomized animals receiving water for injections. A possible explanation is that TNF- $\alpha$  is involved in regulation of even earlier events in liver regeneration [24]. Similarly, the levels of IL-6 were lower in the EGCG50 + PHx group compared to the AI + PHx group.

Furthermore, EGCG was repeatedly reported to activate caspases and increase apoptosis [43–46]. Surprisingly, we found rather lower activity of executive caspases 3/7 in liver homogenates from group V (EGCG50 + PHx) than from animals receiving a lower dose (EGCG20 + PHx) or vehicle only (AI + PHx). An explanation could be the different effects of EGCG on tumor and normal cells: EGCG was shown to protect hepatocytes from apoptosis caused by toxic injury [47, 48]. Direct inhibition of caspase 3 by EGCG was also described [49]. We were not able to detect the Bcl-2 protein using Western blot. However, we are not the first workplace to fail to do so: Tzung et al. [50] reported undetectable levels of Bcl-2 mRNA and protein at 24 h after PHx. Our results of apoptotic body count are also in accord with [50], since the authors showed a peak of

**Fig. 5** Western blot bands of p-p53 protein with beta-actin as a loading control (p21 and p-p27 are not shown as no differences were observed)



high proapoptotic Bad and rather lower expression of antiapoptotic Bcl-x at 24 h after PHx.

Other antiproliferative mechanisms of EGCG are changes in cell-cycle regulating proteins, especially induction of cyclin-dependent kinase inhibitors p21 and p27 [43, 51, 52], and increased expression and serine phosphorylation of p53 [45, 53]. However, we were not able to prove a difference in p21 and p27 expression among hepatectomized groups. Expressions of phosphorylated p53 appeared higher in the EGCG20 + PHx group and surprisingly lower in hepatectomized animals receiving a higher dose of EGCG (EGCG50 + PHx) when compared to the group receiving vehicle (AI + PHx).

Many more antiproliferative mechanisms of EGCG were described, changes in protein kinases, growth factors and transcription factors [38], e.g., inhibition of NF-KB [54].

To check whether the Nrf2 pathway had been activated, we measured the expression of HO-1 and Nqo-1 genes, which belong to Nrf2 effectors, at the mRNA level [55, 56]. However, due to the small number of samples, we were not able to prove activation of this pathway.

Interestingly, EGCG led to higher glycemia compared to vehicle-treated rats, which is in contrary to other findings [22, 23]. It should be mentioned that orally administered EGCG decreases glucose absorption from the intestine, but parenterally administered EGCG hinders glucose uptake into the tissues [57].

Although some antioxidants accelerated liver regeneration after PHx, as could be seen in a study with vitamin C and E [11], and a study with bicyclol [58], other antioxidants and hepatoprotective agents revealed a rather inhibitory effect on liver regeneration after PHx, namely resveratrol [59], curcumin [60] and *S*-adenosylmethionin [61]. Considering the often complex effect of natural antioxidants on cells, the diversity of the results is not surprising. Similarly, the immunosuppressant sirolimus was able to attenuate ischemia–reperfusion injury, but impaired liver regeneration after PHx [62].

Not only our results, but also other studies suggest that EGCG should be used with caution. EGCG is present in over-the-counter extracts from green tea [63, 64] and even in other herbal dietary supplements that do not indicate content of catechins [65]. These supplements are not without a risk—there were repeatedly reported cases of acute liver injury

caused by green tea extracts [66–68]. Although these injuries can be partly explained by toxicity of other substances in the supplements (especially caffeine and/or undetected contaminants), EGCG itself also revealed important effects, particularly interaction with other drugs [69, 70] and a prooxidant effect at high doses [67, 71]. In mice, the dose of 120 mg/kg administered intraperitoneally was hepatotoxic and the dose 150 mg/kg lethal for all mice [71]; the dose of 1,500 mg/kg via a gastric tube was lethal for 85 % of mice [64]. In rats, the dose of 2,000 mg/kg administered via a gastric tube was lethal. Recently, a cholestatic effect of EGCG was described [72]; higher levels of conjugated bilirubin (even though not significantly) in EGCG-treated animals in our study correspond to these findings.

In a study with healthy human volunteers, a single dose of 1,600 mg EGCG taken orally was found to be safe and well tolerated [73]. Potential application of EGCG in clinical studies is complicated by hardly predictable bioavailability [74].

In conclusion, administration of EGCG did not enhance the early phase of liver regeneration after PHx in rats. The higher dose even led to lower DNA synthesis. Although the tests carried out have failed to clarify the mechanism of this effect, it was probably mediated by some pathway other than p53.

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**Conflict of interest** None.

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