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Inhibition of intestinal ischemia/repurfusion induced apoptosis and necrosis via down-regulation of the NF-kB, c-Jun and caspace-3 expression by epigallocatechin-3-gallate administration

ALEXANDROS E. GIAKOUSTIDIS[1,2], DIMITRIOS E. GIAKOUSTIDIS[1], KOKONA KOLIAKOU[5], ELENI KALDRYMIDOU[3], STAVROS ILIADIS[4], NICKOLAOS ANTONIADIS[1], NICHOLAS KONTOS[1], VASILIOS PAPANIKOLAOU[1], GEORGIOS PAPAGEORGIOU[4], KONSTANTINOS ATMATZIDIS[2], & DIMITRIOS TAKOUDAS[1]

[1]*Department of Transplant Surgery,* [2]*Second Department of Surgery,* [3]*Laboratory of Pathology, School of Veterinary Medicine,* [4]*Laboratory of Biological Chemistry, Medical School, and* [5]*Laboratory of Histology and Embryology, Aristotle University and Hippokration Hospital, Thessaloniki, Greece*

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Abstract

Intestinal ischemia/reperfusion (I/R) produces reactive oxygen species (ROS) activating signal transduction and apoptosis. The aim of this study was to evaluate the effect of (−)-epigallocatechin-3-gallate (EGCG) administration in inhibition of apoptosis by attenuating the expression of NF-kB, c-Jun and caspace-3 in intestinal I/R. Thirty male wistar rats were used. Group A sham operation, B I/R, C I/R-EGCG 50 mg/kg ip. Intestinal ischemia was induced for 60 min by clamping the superior mesenteric artery. Malondialdehyde (MDA), myeloperoxidase (MPO), light histology, Fragment End Labelling of DNA (TUNEL), immunocytochemistry for NF-kB, c-Jun and caspace-3 analysis in intestinal specimens were performed 120 min after reperfusion. Apoptosis as indicated by TUNEL and Caspace-3, NF-kB and c-Jun was widely expressed in I/R group but only slightly expressed in EGCG treated groups. MDA and MPO showed a marked increase in the I/R group and a significant decrease in the EGCG treated group. Light histology showed preservation of architecture in the EGCG treated group. In conclusion, EGCG pre-treatment is likely to inhibit intestinal I/R-induced apoptosis by down-regulating the expression of NF-kB, c-Jun and caspase-3.

Keywords: *Ischemia/reperfusion, intestine, epigallocatechin-3-gallate, antioxidants, apoptosis, NF-kB, JKN, c-Jun, caspase-3, MDA, MPO*

Introduction

Restoration of blood supply to the ischemic intestine, resulting in reperfusion injury, remains a significant clinical problem associated with haemorrhage and a state of shock, occurring in patients suffering from coagulopathies, severe trauma, patients undergoing vascular bypass procedures and also intestine transplantation surgery [1–3]. Intestinal ischemia primarily and the consequent bacterial translocation and systemic endotoxin release triggers an activation and accumulation of polymorphonuclear leucocytes and a production of cytokines, adhesion molecules and reactive oxygen species (ROS), initiating an inflammatory response [4–10]. Upregulated

Correspondence: Dimitrios Giakoustidis, MD, PhD, Department of Transplant Surgery, Aristotle University, Hippokration Hospital, Konstantinoupoleos 49, 54642 Thessaloniki, Greece. Tel: +302310889417. Fax: +302310855566. Email: dgiak@auth.gr

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expression of ROS may induce severe oxidative stress due to the imbalance of pro-oxidants and antioxidants, leading possibly to lipid peroxidation [11].

Programmed cell death or apoptosis of intestinal epithelial cells, originally described by Kerr et al. [12] in lymphoid cells, has been documented to play a central role in gut reperfusion injury in experimental models [1,2,9,13]. The phenomenon of apoptosis, which is being investigated thoroughly regarding hepatic ischemia/reperfusion (I/R), includes the initiation phase, with apical caspases being activated, inducing activation of downstream effector caspases, and leading to the execution phase, where morphologic changes become evident, including DNA fragmentation and formation of apoptotic bodies [14–16]. It has been shown that treatment with caspase inhibitors in models of hepatic and intestinal I/R is associated with improved outcome and protection of the ischemic tissue [17–19].

The transcription factor nuclear factor kappa Beta (NF-kB) has been shown to serve as a key regulator of both the hepatic inflammatory as well as regenerative and anti-apoptotic response and also to be activated during I/R of the liver, playing an important role in the gene expression of a large number of pro-inflammatory cytokines, including TNF-α, chemokines and adhesion molecules [20–22]. It has also been documented to be activated during I/R of the intestine, although its specific role still remains unclear [23]. It is assumed that oxidative stress may exert its toxic effect, at least in part, through NF-kB activation [24] and, therefore, antioxidants could work as NF-kB inhibitors, preventing in this way I/R injury. Multiple activated signalling cascades, including signal transducer, p-38 mitogen-activated protein kinases (MAPKs) and stress-activated protein kinase (SAPK) or c-Jun N-terminal kinase (JNK), have been implicated in inflammatory and cell death pathways following I/R of liver [25–28].

Several signal transduction pathways leading to apoptosis have already been described [29], but the activation of c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), a member of the Stress Activated Protein Kinase family, is regarded crucial and leads to phosphorylation of c-Jun and caspase 3 activation [30]. JNK/SAPK activation can be evidently reduced by oxygen-free radical scavengers [31]. According to Fan et al. [32] JNK activation is triggered by free radical species production, implicating the central role of ROS in the molecular and cellular responses to I/R injury.

Green tea extracts deriving from Camellia sinensis have been reported to demonstrate numerous potent properties, including anti-viral, antibacterial, anti-carcinogenic, hypocholesterolemic and antioxidant [33–36]. EGCG has been proved to inhibit morphogenesis of endothelial cells *in vitro*, by down-regulating Ets-1, c-Fos and c-Jun [37]. EGCG is considered to be the most abundant polyphenol in green tea and possibly the most active [33,38,39] and has also been reported to be an efficient scavenger of free radicals and ROS [38], inhibiting iNOS and nNOS activity [40,41] and *in vitro* effectively preventing copper-induced oxidation of LDL [39]. Treatment with green tea extracts in intestinal I/R in rats has been shown to reduce formation of pro-inflammatory cytokines, ICAM-1 and P-selectin [42]. It has also been documented that EGCG inhibited irradiation-induced apoptosis in human HaCaT keratinocytes by inactivating the caspase cascade in these cells [43] and also that it has neuroprotective effects on oxidative-stress-induced apoptosis in neuronal-differentiated PC12 cells [44].

We have previously demonstrated the protective effect of EGCG in intestinal I/R-induced liver and lung injury by inhibition of the lipid peroxidation process [45]. The aim of this study was to evaluate the involvement of the intestinal I/R-induced apoptotic pathway, especially the activation of NF-kB, c-Jun and caspase-3, and the possible protection of the intestine by administration of EGCG in an experimental model of intestinal ischemia and reperfusion injury.

Materials and methods

Experimental procedure

Thirty male Wistar rats weighing 250–300 g were used in this study. The rats were cared for in accordance with the Guide for the Care and Use of Laboratory Animals [46] and kept under a 12-h light/dark cycle and permitted *ad libitum* access to standard laboratory rodent chow and tap water for 2 weeks before the beginning of the experimental procedure.

The animals were divided into three groups. One group was subjected to sham operation. The second was subjected to intestinal I/R and served as the control receiving the same amount of 0.9% normal saline as a vehicle. The third group was subjected to intestinal I/R with intraperitoneal administration of (−)-epigallocatechin-3-gallate (Sigma Aldrich, USA) in normal saline, 15 min prior to ischemia at a dose of 50 mg/kg body weight.

In I/R groups, a midline laparotomy was performed and heparin (Heparin® 5000, Astra Pharmaceuticals, Sweden) was administered intravenously at 50 IU/Kg to prevent thrombosis due to clamping. Intestinal ischemia was induced for 60 min by clamping the superior mesenteric artery with a vascular microclip (Scanlan® International, St. Paul, Minnesota). Removal of the microclip allowed intestinal blood reflow. Following 120 min of reperfusion, the intestine was surgically removed for malondialdehyde (MDA) and myeloperoxidase (MPO), histologic examinations by light, Fragment End Labe*l*ing of DNA (TUNEL assay) and immunocytochemistry.

In the sham operation group, the abdomen was opened, heparin (Astra, Stockholm, Sweden) was administered and the intestine was surgically removed for MDA and MPO assay, light microscopy, examinations and TUNEL assay and immunocytochemistry.

Determination of malondialdehyde (MDA) in intestinal tissue

Determination of MDA, the compound used as an index of lipid peroxidation, was carried out with a selective third-order derivative method [47]. In brief, 1-g samples were thoroughly homogenized (Polytron homogenizer, PCU, Switzerland) with 5 ml of 5% aqueous trichloroacetic acid and 2 ml of 0.8% butylated hydroxytoluene in hexane were added and centrifuged. The top layer was discarded and a 2.5-mL aliquot from the bottom layer was mixed with 1.5 ml of 0.8% aqueous 2-thiobarbituric acid to be further incubated at 70°C for 30 min. Following incubation, the mixture was cooled to room temperature and submitted to conventional spectrophotometry (Shimadzu, Model UV-160A, Tokyo, Japan) in the range 400–650 nm with a scanning speed of 480 nm/min. Third-order derivative spectra were obtained by electronic differentiation (derivative difference setting, 21 nm) of the conventional absorption spectra of samples from both control and drug-treated rats. MDA concentration (nmol/g wet tissue) was calculated on the basis of the third-order derivative peak height at 532 nm by referring to slope and intercept data of the computed least-squares fit of standard calibration curve.

Determination of myeloperoxidase (MPO) in intestinal tissue

Tissue-associated MPO activity was determined by a modification of the method of Krawisz et al. and Grisham et al. [48,49]. Briefly, 1-g samples were homogenized (Polytron homogenizer, PCU, Switzerland) in 10 ml of ice-cold 0.02 m ethylenediaminotetraacetic acid (EDTA), pH 4.7, for 60 s. A 5-ml portion of the tissue homogenate was centrifuged at 20 000 g for 15 min at 4°C and the supernatant, which contained $< 5\%$ of the total MPO activity, was discarded. The pellet was homogenized in 5 ml of potassium phosphate buffer, pH 5.4, containing 0.5% hexadecyltrimethylammonium bromide, centrifuged at 20 000 g for 15 min at 4°C, and the supernatant was collected. A 100-µl portion of the supernatant was mixed with 100 µl of 1.6 mm tetramethylbenzidine in 8% N,N-dimethylformamide, 30 µl of 0.3 mm H_2O_2 and 770 µl of 80 mm potassium phosphate buffer (pH 5.4) to reach a total volume of 1000 µl. The mixture was incubated at 37°C for 3 min, cooled into an ice bath and the MPO activity was assessed spectrophotometrically (Shimadzu, Model UV-160A, Tokyo, Japan) by measuring the H_2O_2-dependent oxidation of tetramethylbenzidine at 655 nm. One unit of enzyme activity was defined as the amount of MPO causing an absorbance change of 1.0/min at a wavelength of 655 nm at 25°C.

Light microscopy examination

Double blind analysis was applied on all intestinal samples. For light microscopy examination, the specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, cut in sections of 5 µm thickness and stained with haematoxylin-eosin.

Fragment end labelling of DNA (TUNEL method)

Apoptosis was estimated by the TUNEL assay, which relies on the incorporation of labelled dUTP at sites of DNA fragments. Sections of 5 µm thickness were deparaffinized and rehydrated in descending alcohol concentrations. The sections were permeabilized with 20 mg/ml proteinase K for 30 min at room temperature and then they were rinsed excessively with PBS, incubated with 3% hydrogen peroxide for 5 min, to inactivate endogenous peroxidase, and rinsed again with PBS. The sections then were covered with parafilm, to avoid drying, and incubated with a mixture containing terminal deoxylnucleotidyl transferase (TdT) enzyme, biotinylated and non-biotinylated deoxynucleotides in an autoclave at 37°C for 1.5 h, according to the manufacturer instructions (Oncogene, Research Products, Cambridge, MA). The reaction was stopped and the sections were incubated with avidin-biotin-peroxidase complex for 30 min, rinsed with buffer and finally the reaction was detected with DAB in the presence of hydrogen peroxide. The sections were rinsed, dehydrated in ascending alcohol concentrations, covered and examined with a light microscope. Positive control slides were prepared in the same way, using HELA cells, that have been pre-treated with actinomycin-D in order to induce apoptosis and negative control slides by omitting the step with TdT enzyme.

Immunocytochemistry

Sections of 5 µm thickness were deparaffinized, rehydrated in descending alcohol concentrations and brought to boiling in 10 mm sodium citrate pH 6.0 for 30 min. Then slides were maintained at subboiling temperature for 10 min and after at room temperature for cooling for a further 30 min. They were then washed with distilled water for 5 min and incubated in 3% hydrogen peroxide for 10 min. Sections were incubated with PBS and then with blocking solution, normal goat serum in dilution 1/5 for 1 h. Sections without washing were incubated with the cleaved caspase-3 (Asp 175) antibody (Cell

Signaling Technology Inc, Danvers, MA) at dilution 1/200 or Phospho-c-Jun (Ser63) II antibody (Cell Signaling Technology Inc, Danvers, MA) at a dilution 1/50 or NF-kB p65 antibody at a dilution 1/1000 (AbCam Ltd., Cambridge, UK) overnight at 4°C. Next day sections were washed thoroughly and incubated with the secondary antibody, goat anti-rabbit biotin conjugated (Sigma-Aldrich, St. Louis, MO), at dilution 1/300 for 30 min at room temperature. Sections then were incubated with extrAvidin peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature at 1/300 and finally were monitor stained closely with DAB tablets (substrate pack, BioGenex Laboratories, San Ramon, CA). After washing, sections were dehydrated in ascending alcohol concentrations, mounted and examined under a light microscope.

Statistical analysis

Overall significance after ischemia and reperfusion was tested by one-way analysis of variance. The homogeneity of the variances was tested using the Levene's test. Differences between means were tested for significance by Duncan's multiple range test [50]. Statistical analyses were performed using the general linear model of SPSS statistical package (SPSS 13, SPSS Ltd., Woking, Surrey, UK).

Results

MDA in intestinal tissue

The animals subjected to I/R displayed a significant increase of MDA levels in intestine compared to the sham operation animals. In the latter, mean MDA level was 0.524 ± 0.15 nmol/g wet tissue, whereas in the I/R animals given normal saline 0.913 ± 0.36 nmol/g wet tissue. Administration of EGCG to the animals prior to I/R resulted in marked reduction of the mean MDA level to 0.565 ± 0.2 nmol/g wet tissue (Figure 1).

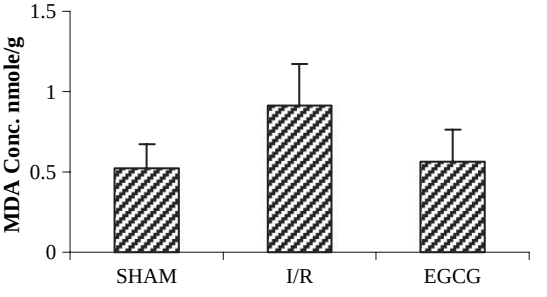

Figure 1. Mean concentrations of MDA in intestinal tissue. In the I/R group (I/R) a statistically significant ($p < 0.05$) increase compared to the sham operation group (SHAM) was observed. On the contrary, in the I/R group treated with EGCG (EGCG) a statistically significant ($p < 0.05$) decrease compared to the I/R group was observed. Values are means of 10 samples $\pm$ SD.

MPO in intestinal tissue

Mean MPO levels in sham operation animals were 1.8 ± 0.21 U/g, whereas animals subjected to I/R displayed increased mean MPO levels, the latter being, 2.92 ± 0.49 U/g. Pre-treatment with EGCG prior to I/R resulted in a marked reduction of mean MPO level to 1.9 ± 0.18 U/g (Figure 2).

Light microscopy examination

In the I/R group, the mucosa of the small intestine has undergone changes to its normal structure. Specifically the villi appear to be contracted and wider. Additionally the epithelial cells on the tips of the villi were flattened and/or desquamated and the number of the goblet cells was increased (Figure 3A). In the I/R group, pre-treated with EGCG, the mucosa of the small intestine appears to be almost normal with thin and tall villi, similar to those of the sham operation group. The epithelial cells were observed to be normal and a number of goblet cells were evident. There can only be detected a small level of apoptosis of the epithelial cells on the tips of the villi (Figure 3B).

Fragment end labelling of DNA (TUNEL method)

In the I/R group almost all of the epithelial cells were positively stained (Figure 4A). In the lamina propria some of the inflammatory cells were positive. In the group pre-treated with EGCG, almost all epithelial cells failed to be positive stained. However, only a few apoptotic cells in the tips of the villi and some positive inflammatory cells were detected in lamina propria (Figure 4B).

Immunocytochemistry

Cleaved caspace-3 (Asp 175). In the I/R group, some cells on the tip of the villi were positive and detached epithelial cells were positive as well (Figure 5A). Inflammatory cells in the lamina propria appeared also to be positive. In the group pre-treated with EGCG only a few positive cells could be detected on

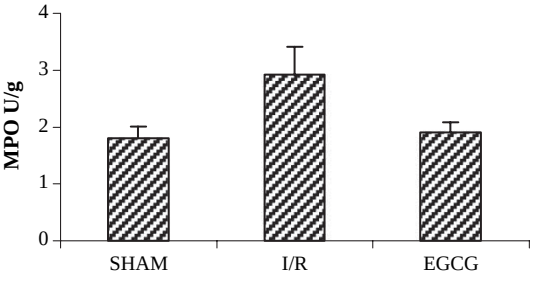

Figure 2. Mean concentrations of MPO in intestine showed a statistically significant ($p < 0.05$) increase in the I/R group (I/R) compared to the sham operation group (SHAM) and the I/R group treated with EGCG (EGCG). Values are means of 10 samples $\pm$ SD.

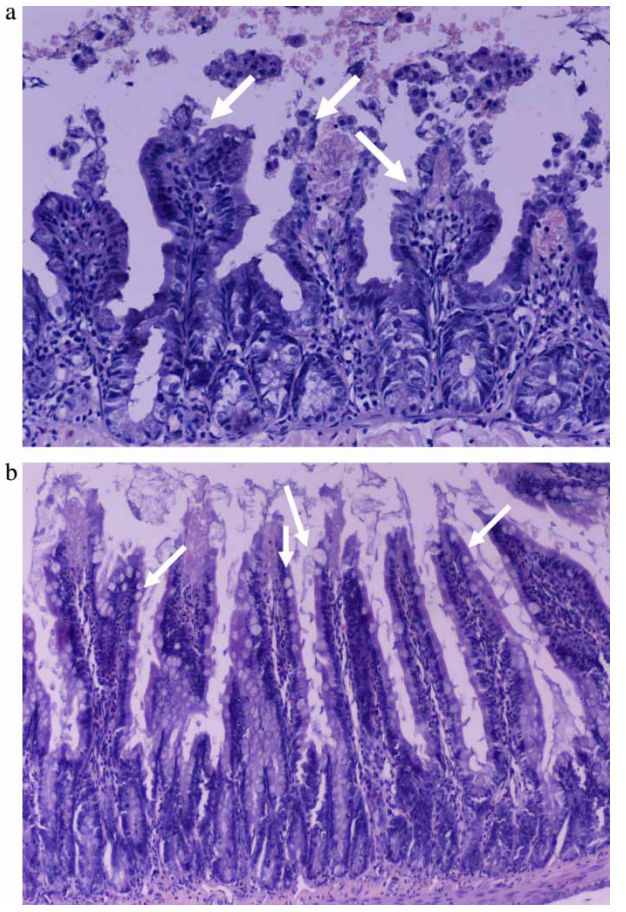

Figure 3. Light microscope photograph of intestinal mucosa. In the I/R group (A), the villi appear to be contracted and wider. Additionally the epithelial cells on the tips of the villi were flattened and/or desquamated (white arrows). In lamina propria inflammatory cells are numerous (×20). In the EGCG group (B), the mucosa of the small intestine appears to be almost normal with thin and tall villi, with evident goblet cells (×10).

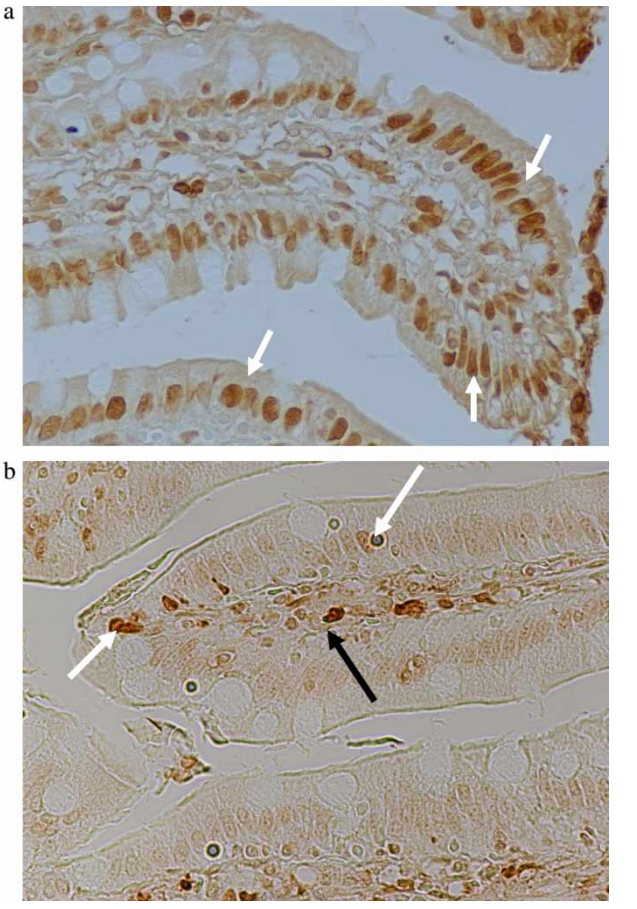

Figure 4. Light microscope photograph of apoptosis of intestinal cells (TUNEL method). In the I/R group (A) nuclei of almost all of the epithelial cells were positively stained (×40). In the EGCG group (B) only a few nuclei of epithelial cells (white arrows) were positive. Although some inflammatory cells in the lamina propria and interepithelial (black arrows) had positive reactions (×40).

the tip of the villi (Figure 5B). The inflammatory cells continued to be positive.

NF-kB p65. I/R specimens exhibit positive stained cytoplasm, especially around the nucleus, and in many cases the nuclear matrix was stained positive as well (Figure 6A). However, in the EGCG group, NF-kB failed to be activated and only fine staining was detected in the cytoplasm (Figure 6B).

Phospho-c-Jun (Ser63). Villi with lesions were observed in the I/R group. In the remaining epithelium, apoptotic cells with positive stained nuclei for c-Jun were detected (Figure 7A). In the group pretreated with EGCG, only a few apoptotic cells with positive stained nuclei could be detected in the tips of the villi, which maintained their normal architecture (Figure 7B).

Discussion

Apoptosis has been implicated to play a key role in the pathophysiological events related to organ transplantation and ischemia reperfusion injury [14,15,51]. It has been described as a programmed type of cell death caused by physiological and/or pathological stimuli including free radicals, TNF-α and growth factor withdrawal [14,52]. Several signal transduction pathways leading to apoptosis have recently been described linking surface receptors such as the Fas or TNF-α receptor with a family of caspases responsible for the initiation of programmed cell death [35].

Fishbein et al. [51] reported the sensitivity of intestinal grafts inherent to transplantation, with increased rates of apoptosis in parallel with increased expression of caspase 3. Previous studies had demonstrated the induction of apoptosis in the small intestinal mucosa following ischemia and subsequent reperfusion [53–55]. Zheng et al. [56] reported attenuation of intestinal apoptosis following administration of a p-38 MAPK inhibitor.

Green tea extracts have been reported to posses antioxidant properties by scavenging ROS and chelating iron/copper activity that could potentially inhibit generation of free oxygen species [35,38–41].

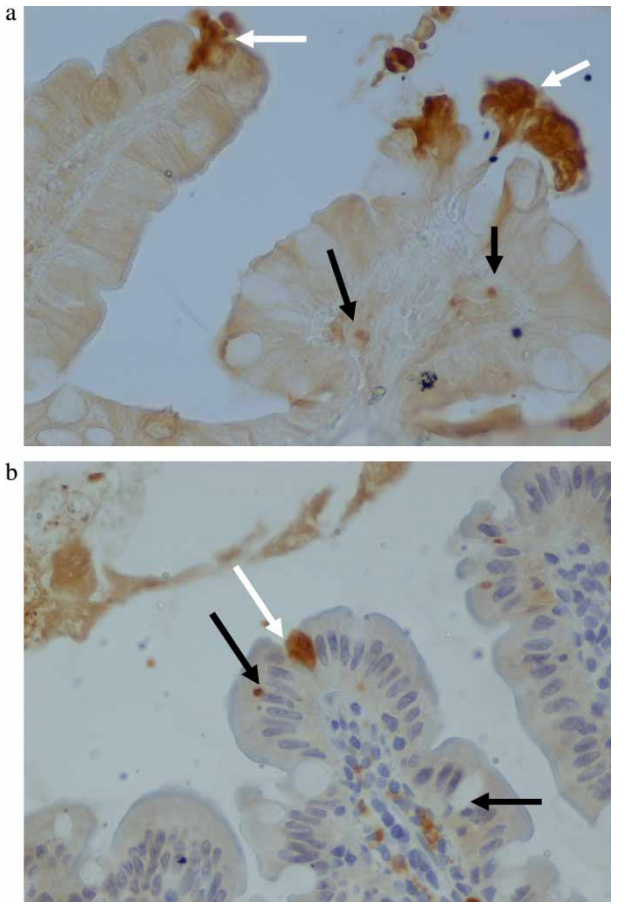

Figure 5. Light microscope photograph of Caspase-3 of intestinal mucosa. In the I/R group some epithelial cells on the tip of the villi were positive (white arrows) as well as some inflammatory cells (black arrows) (A) (×40). In the group pre-treated with EGCG only a few positive epithelial cells could be detected on the tip of the villi (white arrow). Some inflammatory cells in the lamina propria were also stained positive for caspase-3 (black arrows) (B) (×40).

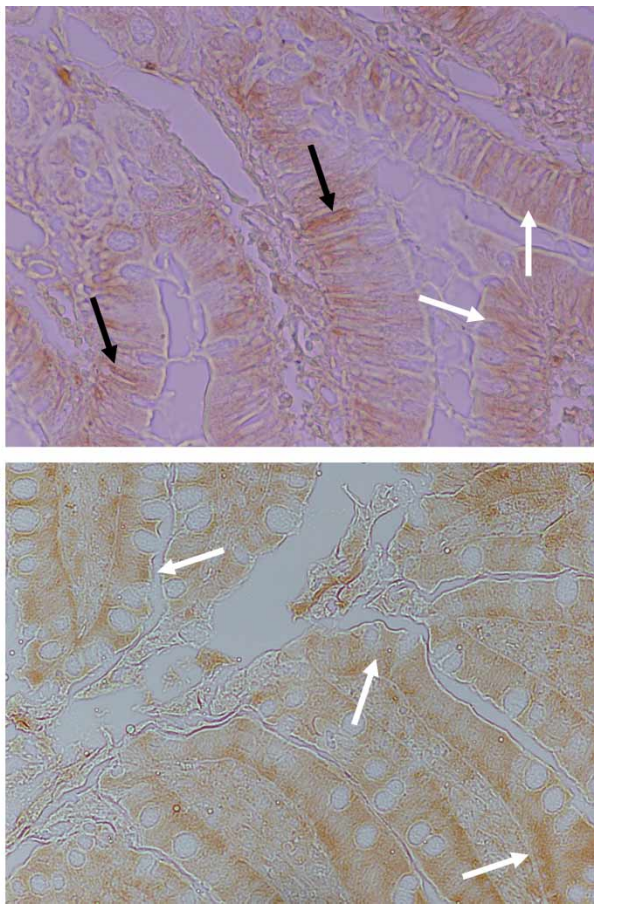

Figure 6. In I/R specimens there were positive stained cells for NF-kB in cytoplasm (white arrows), especially around the nucleus, and in many cases the nuclear matrix was stained positive as well (black arrows) (A) (×40). However, in the EGCG group, NF-kB failed to be activated and only fine staining was detected in the cytoplasm of epithelia cells (white arrows) (B) (×40).

Zhong et al. [57] reported that administration of green tea extracts attenuated warm hepatic ischemia/reperfusion injury and inhibited activation of NF-kB and free radical formation. EGCG has been documented to protect nerve growth factor, differentiated PC12 cells from apoptosis induced by ROS [43] as well as to inhibit irradiation-induced apoptosis of human keratinocytic cell line HaCAT [44].

In the present study it was shown that apoptosis, indicated by TUNEL method, in the group pre-treated with EGCG presented with marked reduction compared to that of the control group, indicating a potential ability of EGCG to attenuate the lethal apoptotic phenomenon following intestinal ischemia/reperfusion. Caspase-3 has a key role in promoting the apoptotic signalling cascade, being an established member of the caspase family and the main downstream effector caspase that cleaves the majority of the cellular substrates in apoptotic cells [58]. Activated caspace-3 was widely expressed in the I/R group, although a very limited amount was detected in the sham operation animals. EGCG appeared able to inhibit activation of caspase-3 in the I/R group pre-treated with EGCG.

Gut barrier impairment holds an important role in I/R injury of small intestine. Philpott et al. [59] identified NF-kB and/or AP-1 activation in the signal transduction pathway in intestinal epithelial cells stimulated with bacteria. According to Fan et al. [32] NF-kB activation and AP-1 increase could be early molecular mediators of the sub-acute inflammatory phase responses. Additionally, Matsushita et al. [60] reported that NF-kB activation by hypoxia induced endothelial cell death and apoptosis through suppression of bcl-2 and activation of caspase-3. On the other hand, Clavien's group reported that bcl-2 over-expression has been shown to protect liver from I/R by inhibiting caspase-3 activation and induction of apoptosis [61] and Guan et al. [28] showed that JNK mediated neuronal death via phosporylation of bcl-2. NF-kB activation and translocation to nucleus has been reported to occur in I/R injury of the liver [32]. Antioxidants could potentially act as NF-kB inhibitors, considering that NF-kB activation may act as a mediator for the toxic effect induced by oxidative

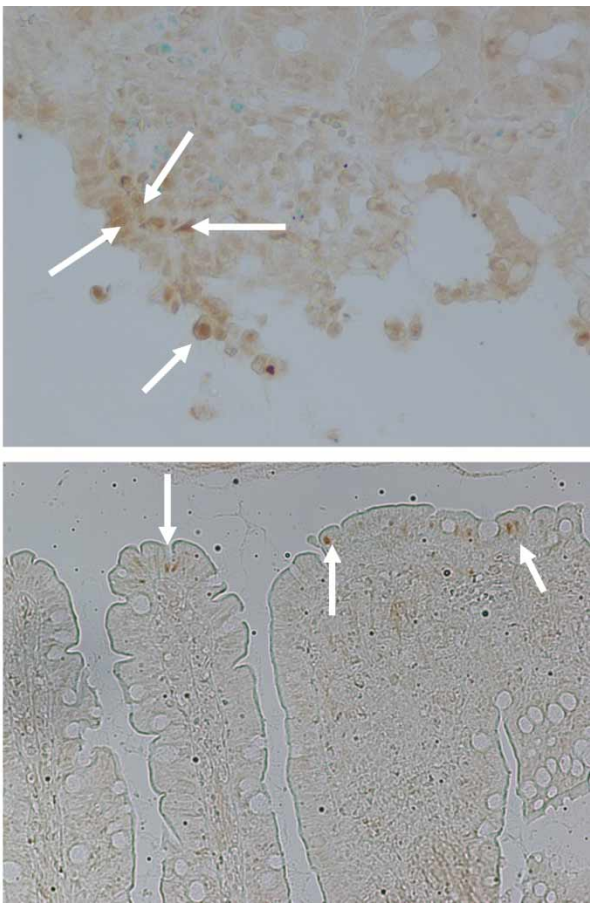

Figure 7. In the I/R group apoptotic cells with positive stained nuclei for c-Jun were detected (white arrows) (A) (×40). In the group pre-treated with EGCG, only a few apoptotic cells with positive stained nuclei could be detected in the tips of the villi (white arrows) (B) (×20).

stress [19]. In the I/R group pre-treated with EGCG, NF-kB activation was attenuated, while it was widely expressed in the I/R group, implicating one pathway of protection from I/R injury to the gut, suggesting either protection to the injured gut by direct inhibiton of NF-kB and/or indirectly by over-expression of BCl-2 and subsequent inactivation of caspase-3.

It has been demonstrated that JNK/SAPK and p-38 MAPK were activated by a variety of cellular stresses, leading to apoptosis and cell death [27,30,56]. Guan et al. [28] reported JNK playing a critical role in ischemia-induced neuronal apoptosis, through c-Jun/AP-1-induced transcription regulation. Brenner's group demonstrated that JNK upregulates liver injury following I/R [25]. Phosporylation and activation of JNK has been reported to be stimulated by a number of stresses including stimulation by cytokines and environmental stresses such as radiation oxidant stress [25]. Crenesse et al. [30,31] reported JNK/SAPK activation leading to apoptosis in hypoxia-reoxygenation of hepatocytes and reduction of its activation with administration of free radical scavengers. Jun aminoterminal kinase (JNK) phosphorylates c-Jun on serines 63 and 73 in response to cellular stress and the transcriptional activity of c-Jun is induced by JNK phosphorylation and JunD is phosphorylated via heterodimerization with either JunB or c-Jun. Intestinal specimens were stained positive for c-Jun in the I/R group. On the other hand the negative intestinal staining for c-Jun in the EGCG treated group could support the hypothesis of failure of JNK activation at reperfusion, leading to failure of transcriptional activity of c-Jun. Aoki et al. [62] have shown that, in conditions of oxidative stress, JNK translocates to mitochondria leading to activation of apoptotic signalling. Considering cytocylic release of cytochrome c may lead to activation of caspase-9 and finally caspase 3 [25], a JNK inhibitor could be able to block also activation of the executioner caspases. EGCG was able to block caspase 3 activation directly or via inhibition of JNK, which was detected via blocking of c-Jun phosporylation in the group pre-treated with EGCG.

Moreover, PMN infiltration as indicated by MPO and lipid peroxidation as measured by MDA both showed significant reduction in the group pre-treated with EGCG, compared with the ischemic group, reaching almost normal values, proving the beneficial antioxidant effect provided by this green tea catechin. Additionally light histology examinations revealed mucosal alterations in the I/R group. In the I/R group pre-treated with EGCG, significant protection of the mucosa of the small intestine occurs and appears to be almost normal with thin and tall villi, similar to those of the sham operation group.

Our results of this study lend support to the hypothesis that apoptosis plays a central role in the deleterious process of reperfusion injury and that blocking the activation of the apoptotic signalling cascade by EGCG pre-treatment may lead to increased protection of the intestine. Additionally, administration of EGCG prior to ischemia/reperfusion is likely to protect the intestine by minimizing lipid peroxidation.

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