Epigallocatechin-3-gallate protects heart and kidney DNA from intestinal ischemia /reperfusion injury in rats

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Abstract
Ischemia/reperfusion (I/R) injury is considered as a major clinical problem. It induced tissue injury and apoptosis mainly via oxygen free radicals. Leading to the development of local and distant organ dysfunction, Objective: This study investigates DNA damage to the heart and kidney, as a result of intestinal I/R, and possible DNA protection through the administration of epigallocatechin-3-gallate (EGCG). EGCG was chosen to be investigated in this model based on previous studies showed its antioxidants, anti-fibrotic and anti-apoptotic effects. Materials and methods: A total of 32 rats were randomly divided into four experimental groups: sham, I/R, I/R pretreated with EGCG (50 mg/kg ip 30 min before ischemia) and EGCG treated (50 mg/kg ip). Apoptosis was assessed using comet assay. Indices of heart and kidney damage were measured (caspase 3, 8-hydroxydeoxyguanosine, Heat-shock protein-70). Results: Intestinal I/R caused heart and kidney damage as noted by significant increased DNA parameters (DNA tailed %, DNA untailed %, DNA Tail length, Tail DNA % and tailed moment), increased serum level of caspase-3,8-hydroxydeoxyguanosine and heat-shock protein-70. EGCG significantly reduced DNA damage parameters on the comet assay in heart and kidney homogenate, and reduced serum levels of caspase-3,8-hydroxydeoxyguanosine and heat-shock protein-70. Conclusion: Based on our results, we conclude that EGCG can protect the heart and kidney against intestinal I/R injury via an anti-apoptotic mechanism.

Introduction
Ischemia reperfusion (I/R) injury of the small intestine is associated with high morbidity and mortality. It can occur following abdominal aortic aneurysm surgery, small bowel transplantation, sepsis, neonatal necrotizing enterocolitis and mesenteric arterial thrombotic or embolic disease (Ramachandran et al., 2000). The small intestine is probably the most sensitive internal organs to I/R induced injury. Moreover, Oxidative stress and free radicals have been shown to contribute to the I/R induced intestinal injury (Mallick et al., 2004). I/R-induced oxygen free radical formation causes oxidative DNA damage and plays a significant role in the pathogenesis of reperfusion injury (Cordis et al., 1998).

Reperfusion of the superior mesenteric artery (SMA) causes activation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). High levels of nitric oxide, proinflammatory and inflammatory cytokines and transcription factors are activated following mesenteric I/R injury and circulate via both the venous and the
lymphatic systems, inducing remote organ injury (Li and Jackson 2002; Kesik et al., 2009). It has been reported that ROS or their oxidation products directly act on mitochondria to induce the opening of the permeability transition pore, followed by the release of pro-apoptotic molecules from mitochondria (Weiss et al., 2003).

Caspase family proteins, especially caspase-3, are known as major apoptosis executors. Pro-caspases are proteolytically cleaved and activated to form active caspases. The activated caspases then cleave various intracellular proteins, such as actin, nuclear lamin and inhibitor of caspase-activated DNase, followed by the induction of apoptotic features, cell shrinkage, chromatin condensation, and DNA fragmentation in the cell. It is generally believed that one of the major functions of heat- shock proteins (HSPs) is to protect cells from the deleterious effects of both physiological and environmental stresses (Li et al., 1992).

The comet assay (single-cell gel electrophoresis) is widely used to assess DNA damage among several signal-transduction pathways leading to apoptosis (Singh et al., 1988).

Epigallocatechin-3 gallate (EGCG), a major component of green tea extracts, has recently been found to have protective role in cardiovascular disease. Several authors showed the beneficial effects of EGCG on cardiac myocytes against ischemia/reperfusion-induced apoptosis and oxidative stress from cardiac hypertrophy (Townsend et al., 2004, Li et al., 2006).

EGCG was chosen to be investigated in this model based on previous studies showed its antioxidants (Giakoustidis et al., 2006), anti-fibrotic (Liang et al., 2011) and anti-apoptotic (Giakoustidis et al., 2010). It has been previously reported that epigallocatechin-3-gallate (EGCG) inhibits irradiation-induced apoptosis in human HaCaT keratinocytes by inactivating the caspase cascade in these cells (Kondo et al., 2004), and that it has neuronal protective effects on oxidative stress-induced apoptosis in neuronal-differentiated PC12 cells (Koh et al., 2003). Giakoustidis and his co-workers (2008) published the protective effect of EGCG in intestinal I/R induced liver and lung injury by inhibiting the lipid per-oxidation process (Giakoustidis et al., 2006). Furthermore, EGCG down-regulated intestinal I/R-induced apoptosis, through blocking the nuclear factor-κB (NF-κB) and c-Jun signaling pathways.

The aim of the present study is to investigate heart and kidney DNA damage induced by intestinal ischemia/reperfusion via ROS-induced apoptosis, and the protective effect of EGCG administration by blocking of HSP-70, 8-Hydroxydeoxyguanosine (8-OHdG) and caspase-3.

Materials and methods

Chemicals and Drugs

EGCG was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Animals and Treatments

Thirty-two adult male Sprague-Dawley rats, weighing 200–250 g, were housed at cages in a temperature-controlled (25°C) environment and provided free access to pellet food and purified drinking water ad libitum. The animal experiments described comply with the ethical principles and guidelines for the care and use of laboratory animals adopted by the National Egyptian Community. Al-Azhar University Committee approved the animal experimental protocols. The rats were divided into four groups (eight in each group) as follows:

Group I: Sham-operated group.
Group II: Intestinal I/R group receiving saline (I/R+ saline).
Group III: Group receiving EGCG 50 mg/kg ip with Intestinal I/R (Giakoustidis et al., 2008) 30 min before ischemia (EGCG + I/R).
Group IV: group receiving EGCG alone 50 mg/kg ip (Giakoustidis et al., 2008).

Induction of intestinal I/R

Abdomen was entered through a midline incision under chloral hydrate anesthesia and I/R rat model was established by method of clamping of superior mesenteric artery for 30 min followed by reperfusion for 60 min (Cheng et al., 2013). Blood was collected from animals by retro-orbital puncture. The animals were killed by cervical dislocation, heart and kidneys were rapidly isolated and washed with ice cold saline. The kidneys and heart were homogenized in KCL. Sham animals were subjected to the same operation as the experimental groups without clamping.

Specimen collection and homogenization
One and half hours after the surgery, blood samples were collected rapidly, serum was obtained by centrifugation. Kidney and heart were removed for comet assay. They were weighed and homogenized in ice-cold 0.15 M KCL using a vertishear tissue homogenizer. The homogenates were centrifuged at 10,000 rpm for 30 min at -4°C, and supernatants were stored at -70°C until Single cell gel electrophoresis (Comet assay) could be performed.

**Single cell gel electrophoresis (Comet assay)**

The Comet assay is a simple and inexpensive method for the detection of different types of DNA damage including single- and double-stranded breaks, DNA adducts, cross-links, and alkaline-labile sites in a low throughout format. For the assay, 0.5 g of crushed samples were transferred to 1 ml ice cold PBS. This suspension was stirred for 5 min and filtered. Cell suspension (100μL) was mixed with 600 μL of low-melting agarose (0.8% in PBS). 100 μL of this mixture was spread on pre-coated slides. The coated slides were immersed in lyses buffer (0.045 M TBE, PH 8.4, containing 2.5% SDS) for 15 min. The slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. Staining with the sample still humid, the DNA fragment migration patterns of 100 cells for each dose level were evaluated with a fluorescence microscope (with excitation filter 420-490 nm (issue 510 nm). The comet's tail lengths were measured from the middle of the nucleus to the end of the tail with 40X increase for the count and measure the size of the comet. For visualization of DNA damage, observations are made of Ethidium Bromide-(EtBr) stained DNA a 40X objective on a fluorescent microscope.

One hundred cells were analyzed on each slide using the Comet assay II automatic digital analysis system (perspective tail length (μm) is the distance of DNA migration from the center of the body of the nuclear core and is used to evaluate the extent of DNA damage). The tail moment is defined as the product of the tail length and the fraction of total DNA in the tail (tail moment=tail length x % of DNA in the tail). Both tail length and tail intensity were measured automatically by image analysis software (Sasaki et al., 1997).

**Immunooassay assessments**

**Caspase 3**
Caspase 3 was determined according to ELISA Kit (Uscn Life Science Inc. Wuhan.) The kit is a sandwich immunoassay in serum of rats’ groups – Cat. No: SEA626Ra.

**Heat Shock protein 70 (HSP-70)**
Heat Shock protein -70 was determined according to ELISA Kit (Uscn Life Science Inc. Wuhan.) The kit is a sandwich immunoassay in serum of rats’ groups- Cat. No: E90873Ra.

**8-Hydroxydeoxyguanosine (8-OHdG)**
8-Hydroxydeoxyguanosine was determined according to ELISA Kit (UscnLife Science Inc. Wuhan.) The kit is a sandwich immunoassay in serum of rats groups- Cat. No: E0660Ra.

**Statistics**
Results are expressed as the mean ± SEM, and the four different groups were compared using one way analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons.

**Results**
DNA tailed %, DNA untailed%, DNA Tail length, Tail DNA % and tailed moment were measured in kidney and heart homogenate of different groups are shown in table 1 and figure 1. There was a significant increase in tailed % in kidney and heart homogenate (19.25±0.854, 13.66±0.623 respectively) in I/R group compared to sham group, while there was significant decrease in group III and VI (6.00±0.707, 10.00±0.408 ) and group VI (12.75±1.031, 4.33±0.881) compared to group II.

There was a significant decrease in un-tailed % in kidney and heart homogenate ( 80.50±0.866, 86.25±0.629 respectively) in I/R group compared to sham group, while there was significant increase in group III and VI (88.25±1.031, 94±0.707 as regard to kidney and 90.00±0.408, 95.67±0.882 as regard to heart) compared to group II.

There was a significant increase in DNA Tail length in kidney and heart homogenate (4.23±0.172, 3.50±0.166 respectively) in I/R group compared to sham group, while there was significant decrease in group III (3.63±0.090, 2.92±0.085) and group VI (1.457±0.093, 1.153±0.040) compare to group II.

There was a significant increase in Tail DNA % in kidney and heart homogenate (4.55±0.172, 3.192±0.264 respectively) in I/R group compared to sham group, while there was significant decrease in group III. 
(4.06±0.055, 2.843±0.154) and VI (1.463±0.067, 1.17±0.178) compare to group II. There was a significant increase in tailed moment in kidney and heart homogenate (19.229±0.103, 11.308±1.014 respectively) in I/R group compared sham group, while there was significant decrease in group III (14.738±0.719, 8.335±0.474) and VI (2.128±0.142, 1.362±0.253) compared to group II. No significant different between group I and group IV in all previous parameters except in kidney tailed %.

Caspase 3, 8-oHDD and SHP-70 are shown in table 2. There was a significant increase in serum in caspase 3, 8-oHDD and SHP-70 in I/R group to 10.055±0.421, 7.800±0.168 and 6.087±0.274 (respectively) compared to sham group. In, I/R EGCG treated group, there was significant decrease to 7.34±0.373, 5.873±0.238 and 4.098±0.084 respectively as compared to I/R group. No significant different difference between group I and group IV.

Figure 1 (1 to 8): Photographs of kidney and heart homogenate of rats analyzed by Comet assay analysis. The “dark/red” round spot represents the intact DNA without migration. The less dark “comet-shaped” area adjacent to the nucleus represents DNA breaks that are small enough to move in the gel. (1—represent kidney of sham group, 2—represent kidney of intestinal I/R group, 3—represent kidney of intestinal treated with EGCG plus I/R group, 4—represent kidney treated with RGCG group, 5—represent heart of sham group, 6—represent heart of intestinal I/R group, 7—represent heart treated with EGCG plus I/R group, 8—represent heart treated with EGCG group.

Table 1: Effect of rats' kidney and heart DNA damage parameters of comet assay
Table 2: Effect of EGCG on rats' serum caspase3,8-OHDG and HSP-70 in intestinal I/R.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Caspase 3 ng/ml</th>
<th>8-OHDG lm/gn</th>
<th>PSH-70 - gm/lm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gp I</td>
<td>3.302 ± 0.303</td>
<td>3.108 ± 0.063</td>
<td>2.238 ± 0.184</td>
</tr>
<tr>
<td>Gp II</td>
<td>10.055 ± 0.421</td>
<td>7.800 ± 0.168</td>
<td>6.087 ± 0.274</td>
</tr>
<tr>
<td>GpIII</td>
<td>7.34 ± 0.373</td>
<td>5.873 ± 0.238</td>
<td>4.098 ± 0.084</td>
</tr>
<tr>
<td>Gp VI</td>
<td>3.063 ± 0.105</td>
<td>2.618 ± 0.117</td>
<td>2.013 ± 0.065</td>
</tr>
</tbody>
</table>

Discussion

Ischemia/reperfusion (I/R) injury represents a source of substantial morbidity and mortality in various statuses that is coronary bypass and myocardial infarction. Oxygen free radicals formed during I/R have been proposed as one of the main causes of tissue injury and play important role in I/R injury (Karahalil et al., 2010).

Oxidative stress occurs when antioxidant defense mechanisms are overwhelmed by free radicals. This imbalance can be caused by either increased free radical formation or decreased antioxidant capacity (Marlin et al., 2004).

Ischemia reduces the activity of cellular defense enzymes against ROS, and reperfusion or introduction of oxygen further disturbs the delicate balance of oxidants/antioxidants, generating a burst of ROS (McCord 1988). Mesenteric I/R is considered to be a triggering event in the development of local and distant organ dysfunction (Souza et al., 2005).

Ischemic/reperfusion neuronal injury is characterized by accumulation of reactive oxygen species and oxidative DNA damage, which can trigger cell death by various signaling pathways (Leak et al., 2013). Apoptosis has been implicated to play a key role in the pathophysiological events related to organ transplantation and ischemia reperfusion injury (Fishbein et al., 2004). Several signal transduction pathways leading to apoptosis have recently been described linking surface receptors such as the Fas or TNF-a receptor with a family of caspases responsible for the initiation of programmed cell death (Higuchi et al., 2003).

Comet assay is a suitable and sensitive method for early detection of low-level DNA damage (Karahalile et al., 2009, 2010). Also, it is a simple, and rapid method in the estimation of DNA damage at the individual cell level through strand breaks, open repair sites, cross-links and labile sites. The assay works on the principle that free radicals, such as ROS, cause breaks in the DNA and/or base oxidation (Hartmann et al., 2003).

Our data indicated that, intestinal I/R group showed significant increase in DNA damage parameters of comet assay represented by tailed %, untailed %, Tail length %, T DNA % and tailed moment as compared to sham group in heart and kidney homogenate.

Our data showed that animals pretreated with EGCG significantly decreased DNA damage as compared to intestinal I/R group.

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 (Munet al., 2005). EGCG is considered to be the most abundant polyphenol in green tea and possibly the most active (Kohri et al., 2001) and has also been reported
to be an efficient scavenger of free radicals and ROS (Lee et al., 2000).
Furthermore, Kondo et al., reported that EGCG inhibited irradiation-induced apoptosis of human keratinocytic cell line HaCaT(Kondo et al., 2004). Therapy with EGCG before reperfusion might exert protective effects via antioxidant activities in a rat experimental model of testicular ischemia-reperfusion injury (Sugiyama et al., 2012). In vivo EGCG treatment rescued the torced testes from IRI-induced inflammation, GCAgene and damage to spermatogonesis thus suggesting a new preventive approach to inhibiting the inflammatory and apoptotic consequences of TT-induced IRI(Al-Maghrabi et al., 2012).
The effect of EGCG on apoptosis in heart and kidney was mentioned previously by many authors. Shenget al., (2007) stated that EGCG inhibits cardiac myocyte apoptosis and oxidative stress in pressure overload induced cardiac hypertrophy. Also, EGCG prevented cardiomyocyte apoptosis from oxidative stress in vitro. Moreover, Sheng et al., (2013) concluded that EGCG, quercetin and carvedilol with potent antioxidant effect, may inhibit cardiomyocyte apoptosis by preventing telomere shortening and telomere repeat-binding factor 2 loss. Liang et al., (2010) demonstrated that PPAR delta agonist and EGCG decreased the Advanced glycation end products - induced kidney cell inflammation and apoptosis.

Giakoustidis et al (2008) finding support the hypothesis that apoptosis plays a central role in the deleterious process of reperfusion injury and that blocking the activation of the apoptotic signaling cascade by EGCG pre-treatment may lead to increased protection of the intestine. The release of cytochrome c in cytoplasm may lead to activation of caspase-9 and finally caspase-3 (Uehara et al., 2005). The 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 (Giakoustidis et al., 2008). Under diverse stress conditions, a highly conserved set of proteins called heat shock proteins has been shown to be synthesized. It is generally believed that one of the major functions of HSPs is to protect cells from the ill effects of both physiological and environmental stresses (Li et al., 1992).
In the kidney, heat shock proteins have also been demonstrated to be protective against oxidative damage (Xiao and Benjamin 1999) and thus provide another avenue for up-regulation of cellular defense systems(Yan et al., 2005). Yan and his co-workers results suggest that HSP25 plays a more basic role in both kidney and heart for cellular redox homeostasis. Induced mitochondrial dysfunction, which a consequence of HSP down-regulation, could be implicated in the cell death and survival pathway(Yan et al., 2005).
Among various forms of DNA lesions, a modified base product, 8-hydroxy-2′-deoxyguanosine (8 ohdG) has been increasingly recognized as an excellent marker of oxidative DNA damage both in vivo and in vitro (Park et al., 1988).
Mitochondrial injury in RTEC leads to the release of apoptogenic factors, including cytochrome c, Smac/DIABLO, Omi/HtrA2, and apoptosis-inducing factor or AIF (Daugas et al., 2000, Servais et al., 2008). The migration of cytochrome c to cytosol is a key event in caspases activation, and the following sequence of events has been described: formation of Apaf-1/cytochrome c apoptosome, caspase-9 activation, and ultimately the activation of the executioner caspase-3(Lee et al., 2001; Park et al., 2002; Cullen et al., 2007).
Taken together, our results showed that Intestinal I/R group significantly increased caspase 3, OHD8G and HSP-70 compared to sham group. However, we found that Pretreatment with EGCG significantly down-regulated the previous parameters as compared to intestinal I/R group.

Conclusions
In summary, the present study demonstrates that EGCG might be particularly useful in protecting against intestinal I/R induced DNA damage in heart and kidney by inhibition of apoptosis and blocking of HSP-70, 8-Hydroxydeoxyguanosine (OHd8G) and caspase-3.

Conflict of interest
No conflicts of disclosure.

References


