Effect of epigallocatechin-3-gallate on inflammatory mediators release in LPS-induced Parkinson's disease in rats

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Degeneration of dopamine (DA)-containing neurons in the substantia nigra of the midbrain causes Parkinson's disease (PD). Although neuroinflammatory response of the brain has long been speculated to play a role in the pathogenesis of this neurological disorder, the mechanism is still poorly understood. The aim of the present study was to examine the effect of epigallocatechin-3-gallate (EGCG) in prevention of inflammatory mediators release and protection of dopaminergic neurons from lipopolysaccharide (LPS)-induced neurotoxicity. A single intraperitoneal injection of LPS (15 mg/kg) in male Sprague Dawley rats resulted in an increase of midbrain content of TNF-α, NO and a decrease of DA level at 4, 24 h, 3 and 7 days compared to the control. In addition, LPS reduced the number and the density of tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the midbrain at 7 days. Pretreatment with EGCG (10 mg/kg) 24 h before LPS for 7 days decreased TNF-α and NO compared to LPS-treated rats. Moreover, it increased DA level and preserved the number and the density of TH-ir neurons compared to LPS group. In conclusion, EGCG was found to have a potential therapeutic effect against LPS-induced neurotoxicity via reducing TNF-α and NO inflammatory mediators and preserving DA level in midbrain.

Keywords: Epigallocatechin-3-gallate, Lipopolysaccharide, Parkinson's disease, Nitric oxide, TNF-α

Parkinson's disease (PD) is a common neurodegenerative disorder affecting 3% of the population over the age of 65. The disease is characterized by cardinal features, including resting tremor, slow movement, rigidity and postural instability as a result of progressive and selective degeneration of dopaminergic neurons in substantia nigra pars compacta (SNpc). The etiology and underlying mechanism responsible for the progressive neurodegeneration of PD remains largely unknown. Increasing evidence suggests that neuroinflammatory responses of the brain, characterized by activation of microglia, might be involved in the pathogenesis of PD.

Microglia, the resident immune cells in the brain, plays a role in immune surveillance and host defense against infectious agents under normal conditions. However, in response to injury, infection or inflammation, microglia become readily activated and produce a variety of proinflammatory factors, including cytokines such as tumor necrosis factor-alpha (TNF-α), the free radical nitric oxide (NO) and superoxide (O₂⁻). Accumulation of these factors is deleterious to neurons.

Lipopolysaccharide (LPS), endotoxin from gram-negative bacteria, causes damage of dopamine (DA) neurons only in presence of microglia. LPS is a potent activator of microglia in various neuron-glia cultures and subsequently causes neuronal cell death. Kim et al. reported that the SNpc relative to other brain regions was more susceptible to LPS-induced neurotoxicity. The underlying mechanism of action by which LPS mediates' neuronal damage is yet unknown.

Certain herbs and plants exhibit antioxidant and anti-inflammatory effects that might be beneficial in reducing neuroinflammatory pathways of the brain induced by LPS. The natural product (-)-epigallocatechin-3-gallate (EGCG) is the major polyphenolic constituent found in green tea (dried fresh leaves of the plant Camellia sinensis).

This polyphenolic compound and several related catechins are believed to be responsible for the health benefits associated with the consumption of green tea. The potential health benefits ascribed...
to green tea and EGCG include antioxidant effects, improving cardiovascular health and enhancing weight loss.10

The aim of the present study is to examine the effect of EGCG in prevention of inflammatory mediators release (TNF-α and NO) and protection of dopaminergic neurons from LPS-induced neurotoxicity in rats.

Materials and Methods

Animals—Adult male Sprague Dawley rats (120) weighing 240-350 g purchased from the Animal House of King Fahad Medical Research Center (KFMRC), King Abdulaziz University (KAU) in Jeddah were used for the study. Rats were kept under standard laboratory conditions with controlled temperature, 12 hr light/dark cycle and given free access to standard diet and water. Animals were acclimatized for one week before starting the in vivo experiment. The experimental protocols were approved by the Faculty Ethical Committee.

Chemicals—The LPS (Escherichia coli-derived; 500,000 endotoxin units/mg) and all chemicals were purchased from Sigma-Aldrich, USA.

Experimental design—The rats were divided into 2 main groups: Group (I): contained 72 rats and was subdivided into 3 subgroups (24 rats each). Subgroup (Ia) was considered as control untreated. Subgroup (Ib) rats received 0.9 % saline vehicle, ip, for 7 days and served as a vehicle. In subgroup (Ic) rats received EGCG (10 mg/kg, ip) for 7 days. Group (II): contained 48 rats. It was divided into 2 subgroups (24 rats each). Subgroup (IIa): rats received a single ip injection of LPS (15 mg/kg), considered as a control for LPS.11 Subgroup (IIb): rats received EGCG (10 mg/kg, ip) 24 h before LPS.12 Injection of EGCG continued for 7 days.

Six rats were randomly selected at 4, 24 h, and 3 and 7 days from each group and anesthetized with sodium pentobarbitone (240 mg/kg, ip).13 Rats were then decapitated, brains were removed and midbrain was dissected on an ice-cold Petri dish. Each dissected midbrain was weighed and stored at -80 °C until time brain tissues were processed for measurement of TNF-alpha, NO and DA.

Tumor necrosis factor- alpha measurement—The amount of TNF-α was measured with a rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit from R & D System (Quantikine, Minneapolis, USA). TNF-α was expressed as pg/mg of tissue.14

Nitric oxide—The level of nitrite (NO2), an indicator of the production of NO, was determined with nitrate/nitrite colorimetric assay kits (Sigma, USA). Nitrite level was expressed as µM/mg of tissue15.

Dopamine—The level of DA was measured using DA ELISA kits (ALPCO, USA). Dopamine level was measured in striatum and expressed as pg/mg of tissue16.

Quantification of nigral tyrosine hydroxylase-positive neurons—Degeneration of dopaminergic neurons was determined by counting the number of tyrosine hydroxylase-immunoreactive (TH-ir) neurons following immunostaining of brain sections.17 Rats were perfused transcardially with PBS followed by ice-cold 4% paraformaldehyde in PBS (pH 7.4). Brains were removed, postfixed for 1 day at 4 °C in 4% paraformaldehyde in PBS and cryoprotected for 2 days at 4 °C in 30% sucrose 1% paraformaldehyde. Coronal sections (4 µm thick) were cut through the nigral complex using a microtome (Thermo Shandon, USA) and stored in PBS containing 0.1% sodium azide. Immunohistochemical staining was performed according to Liu et al.17 Dopaminergic neurons were recognized with an anti-TH polyclonal antibody. Free-floating brain sections were sequentially incubated with the following reagents: 1% H2O2 for 10 min, blocking solution (PBS containing 1% bovine serum albumin (BSA), 0.4% Triton X-100 and 4% appropriate serum) for 40 min, primary antibody diluted in blocking solution, anti-TH, 1:20,000, overnight at 4 °C, biotinylated secondary antibodies, 1:227 in PBS containing 0.3% Triton X-100 for 2 h and vectastain avidin-biotin complex (ABC) reagents for 40 min. Sections were washed two or three times in between steps. The bound antibody complex was visualized with 3,3’-diaminobenzidine. The images were analyzed with Olympus Bx 51 connected with pro-image analysis software. Three sections were measured per group. Quantification of the nigral TH-positive neurons was performed by visually counting of the number of nigral TH-positive neuronal cell bodies under a microscope by three individuals in a blind fashion.

Statistical analysis—All experiments were performed at least thrice. Data were presented as mean±SD and percentage change was calculated. SPSS for windows (version 12) was used for statistical analysis. Experimental data were analyzed with one way analysis of variance (ANOVA) followed by LSD. A value of P < 0.05 was considered statistically significant.
Results

Effect of EGCG on LPS-induced tumor necrosis factor-alpha release—Intraperitoneal LPS (15 mg/kg) injection resulted in an increase in TNF-α levels in the midbrain compared to control. A significant amount of TNF-α was detected within 4 h after injection of LPS, marked increase in TNF-α was seen at the 24 h then a gradual decrease at 3 and 7 days (Fig. 1).

Injection of saline in rats showed no significant effect on the production of TNF-alpha at all the times compared to the control. Injection of EGCG (10 mg/kg, ip) in LPS-treated group caused decrease in TNF-α throughout the study period.

Treatment with EGCG significantly reduced the concentration of TNF-α at 4 h by 92.9% compared to LPS-treated groups. The level of TNF-α 24 h after EGCG administration was significantly decreased by 74.2 % compared to LPS-treated rats (Fig. 1a). Pretreatment with EGCG decreased LPS-induced TNF-alpha release at 7 days by 89.6% compared to LPS-treated group.

Effect of EGCG on LPS-induced nitrite—Nitrite accumulation was used as an indicator of nitric oxide production in the tissue. Intraperitoneal administration of LPS produced significant increase in NO₂ levels in the midbrain at all time points compared to the control (Fig. 1b). The levels of NO₂ in response to systemic LPS increased gradually in the midbrain along 7 days. Injection of saline in rats showed no significant effect on the production of NO₂ at all the times compared to the control.

Treatment with EGCG showed significant decrease in NO₂ levels in midbrain as compared to LPS-treated groups. After 4 h of ip administration of LPS, the levels of NO₂ in the midbrain were significantly decreased in EGCG-treated rats by 36.9 % compared to LPS-treated rats. After 24 h of ip injection of LPS, there was a significant decrease in NO₂ level in the midbrain of EGCG-treated rats by 40.1% as compared to LPS-treated rats (Fig. 1b). Administration of EGCG resulted in significant decrease in NO₂ level at 3 days by 48.2% compared to LPS group. Treatment with EGCG showed significant decrease in NO₂ levels in the midbrain at 7.

Effect of EGCG on LPS-induced loss of dopamine—Rats receiving LPS injection (ip), showed a significant reduction in DA levels of the midbrain compared to the control rats. The levels of DA in response to systemic LPS decreased gradually throughout the study period (Fig. 1c). Treatment with EGCG prevented the decrease in midbrain DA levels induced by LPS. Dopamine levels were significantly high in EGCG-treated rats at 4 hr (1118 ± 0.9 pg/mg) compared to (984.5 ± 1.1 pg/mg) in the LPS-treated rats (Fig. 1c).

After 24 h of administration of LPS, the levels of DA in the midbrain were significantly high in the EGCG-treated rats as compared to those in the
LPS-treated rats. The levels of DA were significantly high at 3 and 7 days in EGCG-treated rats as compared to LPS-treated rats.

**Effect of EGCG on LPS-induced loss of dopaminergic neurons**—Immunohistochemistry for TH-ir neurons in the midbrain at 7 days was conducted to determine the effect of EGCG on LPS-induced loss of dopaminergic neurons in rats. LPS treatment caused significant loss (74.9%) of TH positive neurons in the midbrain compared with those of control animals (Table 1). When EGCG was administrated to rats, dopaminergic cell count was increased significantly.

In addition to the increase in cell numbers, TH-ir neurons in EGCG-pretreated group displayed more extensive density compared with those in LPS group. The density of TH-ir neurons decreased in LPS-treated rats by 90.9% compared to the control. However, EGCG significantly prevented LPS-induced reduction in the density of TH-ir neurons as compared to that in the LPS group at 7 days.

**Discussion**

One of the etiological factors of PD is microglial activation of the brain. In the present study LPS is used to induce neuroinflammatory response of the brain in vivo. Dopaminergic neurons in SN are thought to be more susceptible to inflammation when compared to other neuronal subtypes and other brain regions. Herrera et al. reported that a single injection of LPS produced a strong inflammatory response in SN and leads to damage of only the dopaminergic neurons, with no detectable affect to either the GABAergic or the serotonergic neurons. Microglial activation has been shown to induce the selective loss of dopaminergic neurons in response to multiple stimuli, including LPS. Additionally, the SN contains 4.5 times as many microglia when compared to other brain regions, making this region particularly susceptible to inflammatory insult.

The present study examined the possible neuroprotective effect provided by EGCG on LPS-induced inflammation and neurodegeneration of dopaminergic neurons in vivo.

Neurodegeneration was analyzed in vivo where systemic administration of LPS to adult male rats release significantly the TNF-α and NO levels in the midbrain at 4, 24 hr, 3 and 7 days. TNF-α concentration in response to systemic LPS peaked in the midbrain at 24 hr and decreased gradually at 3 and 7 days while NO levels increased throughout the study. In addition, intraperitoneal LPS injection showed a significant decrease in dopamine level of the midbrain gradually and results in significant loss of TH-ir neurons in the midbrain at 7 days by 74.9% compared with control. The density of the TH-ir cells decreased in LPS-treated rats by 90.9% at 7 days compared to control.

The present results are supported by Singh and Jiang, reporting that LPS (100 µg/kg) when injected systemically in rat triggers a series of signaling events leading to the development of inflammatory response in the brain. Other findings demonstrated that systemic LPS activates kupffer cells, the resident macrophage-like cells in the liver to produce TNF-α, which is distributed in the blood and transferred to the brain through TNF-alpha receptors to induce the synthesis of additional TNF-α and other pro-inflammatory factors, creating a persistent and self-propelling neuroinflammation that induces delayed and progressive loss of dopaminergic neurons in SN of adult animals.

Release of NO in response to LPS in the present study could be explained on the basis of results of Mayhan that TNF induces NO production which disrupts the blood-brain barrier (BBB). According to Pan et al., TNF independently of NO may have a direct effect on the morphology of endothelial cells and on the permeability of the BBB. TNF may facilitate migration of leukocytes into the central nervous system by enhancing adhesion between leukocytes and endothelial cells, which is a characteristic of inflammation.

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**Table 1**—Effect of EGCG on LPS-induced loss of TH-immunoreactive neurons in rat midbrain

[Values are mean ± SD. Figures in parenthesis are % decrease over control values]

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of TH positive neurons</th>
<th>Density of TH positive neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>867 ± 1.1*</td>
<td>16.4 ± 1.09*</td>
</tr>
<tr>
<td>LPS (15 mg/kg)</td>
<td>218 ± 1.08†</td>
<td>1.5 ± 1.1†</td>
</tr>
<tr>
<td>LPS and EGCG (10 mg/kg)</td>
<td>444 ± 1.11#</td>
<td>8.35 ± 0.9†*</td>
</tr>
</tbody>
</table>

Significant change from †control; #LPS. The percent change was compared to the control. Significance was P< 0.05.
Other findings demonstrated that 5 mg/kg systemic LPS can actively induce persistent inflammation and progressive neurotoxicity over 10 months in the adult mice\textsuperscript{25}. A previous study carried by Ling et al.\textsuperscript{26} showed that the density of the TH-ir cells decreased with cell loss in the rats exposed to a single intraperitoneal injection of LPS (1 mg/kg).

The results of the present study demonstrated that EGCG (10 mg/kg, ip) significantly diminished the TNF-\(\alpha\) and nitrate levels in LPS-treated rats at 4, 24 hr, 3 and 7 days. Treatment with EGCG showed significant increase in DA level in the midbrain and increase in TH-ir neurons in the midbrain at 7 days.

The inhibitory effect of EGCG on LPS-induced release of proinflammatory factors could be attributed to the anti-inflammatory effect of EGCG\textsuperscript{27}. LPS produces inflammation through activation of protein kinase C, which implicated in hydroxyl free radical formation\textsuperscript{28} and nuclear factor kappa-B (NF-\(\kappa\)B) activation\textsuperscript{29}. NF-\(\kappa\)B is a mammalian transcription factor that controls a number of genes that are important for immunity and inflammation\textsuperscript{30}. NF-\(\kappa\)B is composed mainly of two proteins: p50 and p65. In its unstimulated form, NF-\(\kappa\)B is present in the cytosol bound to the inhibitory subunit of NF-\(\kappa\)B (I\(\kappa\)B). After induction of cells by a variety of agents, I\(\kappa\)B becomes phosphorylated and triggers a proteolytic degradation of I\(\kappa\)B; then, NF-\(\kappa\)B is released from I\(\kappa\)B and translocated to the nucleus\textsuperscript{31}. However, EGCG exerts its anti-inflammatory effect through inhibition of LPS-induced phosphorylation and degradation of I\(\kappa\)B. EGCG inhibited the signal transduction pathway of NF-\(\kappa\)B activation through inhibition of LPS interaction with its receptor, scavenging reactive oxygen species (ROS) and inhibition of protein kinases. In addition, EGCG inhibit nNOS in the substantia nigra\textsuperscript{32}. Another possibility of the neuroprotective effect of EGCG could be free radical scavenging and antioxidant effect\textsuperscript{33}.

Green tea polyphenols (10 to 30 \(\mu\)g/mL) increase dopamine, through which they block neurotoxin 1-methyl-4-phenylpyridinium (MPP\(^+\)) uptake and protect dopaminergic neurons against MPP\(^+\)-induced injury\textsuperscript{34}.

In summary, EGCG may be a good candidate for neurodegenerative disease. Further understanding of the mechanisms of action would provide important insights into potential therapeutic interventions for inflammation-related neurodegenerative diseases. It is recommended that regular consumption of EGCG may help to reduce incidence of Parkinsonism.

Acknowledgment

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References

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