New approach in treatment of brain injury: Neurotrophic effects of Apigenin

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ABSTRACT

Brain injury initiates a neuroinflammatory cascade that contributes to substantial neuronal damage. Administration of lipopolysaccharide (LPS) impaired antioxidant mechanisms, increased peroxidation and impaired mitochondrial redox activity causing brain inflammation as well as neuronal damage and impairment of brain monoamines. Apigenin gathered extensive attention in recent years because of its chemopreventive, antioxidant and anti-inflammatory effects. This study aimed to evaluate the impact of apigenin in LPS induced brain injury in experimental rats and to evaluate its role in monoamines regulation as well as DNA damage reduction. Forty male albino rats were used in this study, divided into four groups (control, apigenin, LPS and treated groups). Brain malondialdehyde (MDA), brain nitric oxide (NO) and serum paraoxnase activity (PON-1) were estimated colorimetrically. DNA damage was evaluated by comet assay method, in addition to brain monoamines assessment by HPLC. Histopathological and Immunohistochemistry of cyclooxygenases (COX-1, COX-2) were also performed. The data showed that lipopolysaccharide significantly increased brain MDA, NO and monoamines concomitant with a reduction in PON-1. Contrarily, apigenin supplementation improved these values in treated group. The present study provides insights into the design of flavonoid with optimal neuroprotective activities.

Keywords: Brain injury, apigenin, neurotransmitters, HPLC, DNA damage, cyclooxygenases

INTRODUCTION

Brain injury is defined as a damage to the brain resulting from an external mechanical force, which can lead to temporary or permanent impairment of cognitive, physical and psychosocial functions [1].

In such case, there are significant increases in oxidative stress [2,3], ionic imbalances, ATP depletion, excitotoxicity, and proteolysis [4]. A close relationship was found between the degree of oxidative stress and the pathogenesis of brain injury [5]. Enhanced reactive oxygen/nitrogen species (ROS/RNS) causes oxidative/nitrosative stress in brain injury leading to damage in lipids, proteins, and nucleic acids [6].

Bacterial LPS is widely used in experimental animals to study the effect of peripheral inflammatory stimuli on brain functions. Administration of LPS has been shown to impair antioxidant mechanisms, increase peroxidation and impair mitochondrial redox activity [7,8] causing brain inflammation [9] as well as neuronal damage [10] and impairment of brain monoamines.

Monoamines have various functions in the brain, including playing important roles in behavior and cognition, such as motor activity, motivation, regulation of milk production, sleep, mood, attention, and learning [11].
Clinical trials in humans following brain injury have not been shown to be very effective. One of the primary reasons is that most trials focus on a single aspect of the cascade. It is now clear that, some types of pharmacologic intervention with a multifaceted approach, addressing multiple secondary injury targets in a complimentary way, will be necessary [12].

Flavonoids are important metabolites of vascular plants structurally related to flavones. The flavonoid apigenin is present in different plants (*Matricaria, Passiflora, Perilla*) and gathered extensive attention in recent years because of its chemopreventive effects [13], antioxidant effects, free radical scavenging, antimutagenic, anti-inflammatory and antiviral effects [14].

From this point of view, we aimed to evaluate the impact of apigenin in lipopolysaccharide induced brain injury in experimental rats and to evaluate its role in monoamines regulation as well as DNA damage reduction.

**EXPERIMENTAL SECTION**

**Materials**

**Chemicals**

Norepinephrine, dopamine and serotonin, high performance liquid chromatography (HPLC) standards, and lipopolysaccharide were purchased from Sigma Aldrich Chemicals Company (St Louis, Missouri, USA). All other chemicals (HPLC grade) were purchased from Sigma.

**Experimental animals**

Sixty male albino rats weighing 120–150 g were obtained from the animal house of the National Research Centre (NRC), Giza, Egypt. The animals were housed in individual suspended stainless steel cages at the temperature range of 22 ± 2 °C, under a 12-h light/12-h dark cycle, and allowed to acclimatize for a period of 10 days to the experiment; rats were allowed free access to food and water. The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of National Research Centre.

**Plant**

*Petroselinum sativum Hoffm* (parsley) seeds were purchased from local market.

**Methods**

**Isolation and preparation of apigenin 7-O-β-glucoside**

Dried powder of parsley seeds, *Petroselinum sativum Hoffm.*, was placed in a continuous Soxhlet extraction apparatus and subjected to successive extraction using petroleum ether (40°C - 60°C), followed by 70% aqueous methanol. The solvent of each extract was evaporated under vacuum to dryness. The aqueous methanol extract was concentrated to a small volume and partitioned successively with chloroform and ethyl acetate. The ethyl acetate fraction was subjected to a polyamide 6S column, then Sephadex LH-20 using methanol where apigenin 7-O-β-glucoside was isolated and identified by comparing its NMR spectral data with those reported in literature [15].

**Experimental design**

Forty male albino rats were classified into four groups (10 rats in each group) as follows:

Group I (control group): healthy rats, received a vehicle.

Group II (apigenin group): healthy rats, received apigenin dissolved in a vehicle. (0.78 mg /kg body weight / day) orally for ten days.

Group III (LPS group): healthy rats, received a vehicle for ten days followed by intraperitoneal (i.p.) injection of lipopolysaccharide (LPS: 100 mg/kg body weight) [16].

Group IV (treated group): healthy rats, received apigenin (0.78 mg /kg body weight / day) orally for ten days followed by intraperitoneal (i.p.) injection of lipopolysaccharide (LPS: 100 mg/kg body weight).

Blood was withdrawn (after four hours of LPS injection) from the retro-orbital venous plexus of the eye using capillary tubes and collected in clean dry tubes for serum separation and in tubes contain EDTA for comet assay. Brain was removed quickly and washed with ice-cold saline solution (0.9% Na-Cl) and divided into two parts; the first part was homogenized and prepared for biochemical estimation; the other part was prepared for histopathological and immunohistochemical studies.

**Preparation of tissue homogenate**

Tissues were cut into small pieces and homogenized in phosphate buffer (pH 7.4), centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant was removed for chemical parameters estimation [17].
Biochemical analysis:

Determination of brain lipid peroxidation

Lipid peroxidation was assayed by measuring the level of MDA in the brain tissues. Malondialdehyde was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea et al, in which the thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid to produce a red colored complex having peak absorbance at 532 nm [18].

Determination of brain nitric oxide level

NO measured as nitrite was determined by using Griess reagent, according to the method of Moshage et al. [19], where nitrite, stable end product of nitric oxide radical, is mostly used as indicator for the production of NO.

Determination of serum paraoxonase activity

The arylesterase activity of paraoxonase was measured spectrophotometrically in supernatants using phenylacetate as a substrate. In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate, resulting in phenol formation. The rate of phenol formation is measured by monitoring the increase in absorbance at 270 nm at 25 ºC. The working reagent consisted of 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM CaCl2 and 4 mM phenyl acetate, as the substrate. Samples diluted 1:3 in buffer were added and the change in absorbance was recorded following a 20-s lag time. Absorbance at 270 nm was taken every 15 s for 120 s using UV Spectrophotometer [20].

Comet assay

Comet assay has been developed to detect cellular DNA damage as described by Singh et al. [21] after modification of the method of Blasiak et al. [22].

Lymphocytes were isolated and washed by phosphate-buffered saline (PBS) at pH 7.4. Ten µl of the cells were suspended in 75 µl of 0.5% low melting agarose for pipetted on microscopic slides with a layer of 1% agarose, spread using a coverslip and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the coverslip, the slides were immersed in cold lysis solution for 1 h, followed by electrophoresis at 25 V, 300 mA, for 40 min. then, the slides were gently removed from the tank and washed three times with 0.4 M Tris base at pH 7.5 for 10 min. Twenty µl from ethidium bromide (10 µg/ml) was added to each slide.

Visualization and analysis of Comet Slides

The slides were examined at 40× magnification using fluorescence microscope (Leica Microsystems, CMS GBh, Wetzlar, Germany, Model DM 2500) and power Max. 160 W. equipped with an excitation filter of 549 nm and a barrier filter of 590 nm. Damaged cells were visualized by the “comet appearance”, with a brightly fluorescent head and a tail to one side formed by the DNA containing strand breaks that were drawn away during electrophoresis. Samples were analyzed by counting the damaged cell out of 100 cells per slide to calculate the percent of damage.

Determination of brain monoamines

Determination of brain norepinephrine, dopamine and serotonin was carried out as described previously by Hussein et al. [17] using high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (G131A model).

Separation was achieved on ODS-reversed phase column (C18, 25 x 0.46 cm i.d. 5 µm).

The mobile phase consisted of potassium phosphate buffer/methanol 97/3 (v/v) and was delivered at a flow rate of 1.5 ml/min. UV detection was performed at 270 nm, and the injection volume was 20 µl.

The concentration of both catecholamines and serotonin were determined by external standard method using peak areas. Serial dilutions of standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations of each standard. The concentration in samples was obtained from the curve.

Histopathological investigations

The brains were fixed in buffer formalin for 24 hours. The specimens were washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin wax (melting point 55-60 ºC). Sections of 6µm thickness were prepared and stained with Haematoxylin and eosin [23]. In this method the paraffin sections were stained in Harris’s haematoxylin for 5 minutes. Sections were washed in running water for blueing and then stained in 1% watery eosin for 2 minutes, washed in water, dehydrated, cleared and mounted in Canada balsam.
Immunohistochemistry of cyclooxygenases

From 10% formalin fixed paraffin embedded samples, 5µm-thin sections were prepared on positive charged slides. The sections were deparaffinized and treated with 0.2% saponin (Thermo Fisher Scientific, Fremont Blvd, USA) at room temperature for 30 minutes. After the sections were treated with methanol containing 3% hydrogen peroxide for 15 minutes to eliminate endogenous peroxidase, the sections were reacted with 10% normal rabbit serum for 10 minutes to block nonspecific reactions. As the primary antibody, each of anti-cyclooxygenase-1 and anti-cyclooxygenase-2 polyclonal antibodies (Thermo Fisher Scientific, Fremont Blvd, USA) were diluted 100 times and reacted with the sections at 4 °C for 15 hours. After the streptavidin–biotin complex method (Thermo Fisher Scientific, Fremont Blvd, USA), biotin-labeled anti-goat immunoglobulin G antibody as the secondary antibody was reacted with the sections at room temperature for 15 minutes, and the peroxidase-labeled streptavidin was reacted at room temperature for 10 minutes, followed by color development using diaminobenzidine (DAB) reagent.

After counterstaining with hematoxylin, the sections were observed under a microscope.

Statistical analysis

All data were expressed as mean ± standard error. Data were analyzed using one-way ANOVA using SPSS (Version 16). Duncan’s new multiple-range test was used to assess differences between means. A significant difference was considered at the level of P < 0.05.

RESULTS AND DISCUSSION

The generation of reactive oxygen species (ROS) in normal cells is controlled by biological antioxidants and antioxidant enzymes. In pathophysiologic conditions, the generation of oxidants exceeds the intracellular antioxidant capacity, resulting in oxidative damages to proteins, lipids and DNA.

In the present study, intraperitoneal injection of lipopolysaccharide in a dose of (100 mg/kg body weight) significantly increased MDA, NO and DNA damage (P = 0.00) concomitant with a reduction of PON-1 (P = 0.00) (table 1, figure 1).

<table>
<thead>
<tr>
<th>Parameters / Groups</th>
<th>PON-1 IU / ml</th>
<th>NO µmol/g tissue</th>
<th>MDA nmol/g tissue</th>
<th>DNA damage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group Mean ±SE</td>
<td>471 ± 35</td>
<td>9 ± 2</td>
<td>300 ± 4.7</td>
<td>7.5 ± 5</td>
</tr>
<tr>
<td>Apigenin group Mean ±SE</td>
<td>477 ± 20</td>
<td>6.1 ± 1</td>
<td>306 ± 8</td>
<td>7 ± 1</td>
</tr>
<tr>
<td><em>P</em></td>
<td>N.S.</td>
<td>.000</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>LPS group Mean ±SE</td>
<td>283 ± 11</td>
<td>15 ± .7</td>
<td>404 ± 13</td>
<td>39.5 ± 2.5</td>
</tr>
<tr>
<td><em>P</em></td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td>Treated group Mean ±SE</td>
<td>452 ± 24</td>
<td>5.6 ± 2</td>
<td>254 ± 23</td>
<td>31 ± 2</td>
</tr>
<tr>
<td><em>P</em></td>
<td>N.S.</td>
<td>.000</td>
<td>.04</td>
<td>.000</td>
</tr>
<tr>
<td><em>P</em></td>
<td>.000</td>
<td>.000</td>
<td>.010</td>
<td>.010</td>
</tr>
</tbody>
</table>

Significant value ≤ 0.05
*P* value compared to control group.
*P* value compared to LPS group.

Increased generation of nitric oxide can be deleterious to brain functions, where synthesis of nitric oxide by both the inducible and constitutive nitric oxide synthase isoforms contributes to the activation of apoptotic pathways in the brain during systemic inflammation induced by LPS [24].

This inflammation was confirmed in our study by assessment of COX-1 and COX-2 by Immunohistochemical technique. In lipopolysaccharide group, brain cerebrum showed negative reaction of Cox 1 (figure 3- C). Whereas, examination of sections in brain cerebrum of control and apigenin groups showed positive reaction of Cox 1 as indicated by the presence of the brown color (figure 3- A, B).On the other hand, supplementation of apigenin in treated group showed positive reaction (figure 3- D).

Contrarily, examination of sections in brain cerebrum of control and apigenin groups showed negative reaction of Cox 2 as indicated with the absence of the brown color (figure 4- A, B).
In lipopolysaccharide group, brain cerebrum showed positive reaction of Cox 2 that appears as brown color (figure 4- C). On the other hand apigenin supplementation in the treated group showed little amount of brown color indicated the reduction of Cox 2 reaction (figure 4- D).

Table 2. Brain monoamines levels in different studied groups

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Norepinephrin (µg/g tissue)</th>
<th>Dopamine (µg/g tissue)</th>
<th>Serotonin (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>3.5 ± .2</td>
<td>3.2 ± .1</td>
<td>3.2 ± .1</td>
</tr>
<tr>
<td>Apigenine group</td>
<td>Mean ±SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SE</td>
<td>2.5 ± .08</td>
<td>2.9 ± .5</td>
<td>2 ± .2</td>
</tr>
<tr>
<td>P</td>
<td>.005</td>
<td>N.S.</td>
<td>.003</td>
</tr>
<tr>
<td>LPS group</td>
<td>Mean ±SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SE</td>
<td>6 ± .2</td>
<td>6 ± .8</td>
<td>4 ± .1</td>
</tr>
<tr>
<td>P</td>
<td>.000</td>
<td>.006</td>
<td>.01</td>
</tr>
<tr>
<td>Treated group</td>
<td>Mean ±SE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SE</td>
<td>2.5 ± .4</td>
<td>1.9 ± .18</td>
<td>1.1 ± .1</td>
</tr>
<tr>
<td>P</td>
<td>.005</td>
<td>N.S.</td>
<td>.000</td>
</tr>
<tr>
<td>P</td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
</tr>
</tbody>
</table>

Significant value ≤ 0.05
P<sub>a</sub> value compared to control group.
P<sub>b</sub> value compared to LPS group.
Histopathological results
COX-2- derived prostaglandin (PG) promotes inflammation by increasing vascular permeability and vasodilatation and by directing the synthesis and migration of pro-inflammatory cytokines into the site of inflammation [25]. The mitogenic and pro-inflammatory functions of COX-2 are linked primarily to exaggerated synthesis of PGE2 [26].

All these conditions resulting in a significantly increased ($P \leq 0.00$) of brain monoamines (dopamine, norepinephrine and serotonin) levels in LPS group compared to control, however, apigenin supplementation significantly decreased these values ($P \leq 0.05$) compared to LPS group, also in comparison to control group except for dopamine which is insignificantly changed compared to control group (table 2).

![Figure 2](image-url)  

**Figure 2.** Micrographs of sections in brain cerebrum of A): control shows the highly active nerve cells (neurons). B): apigenin group shows the normal structure of cerebrum. C): lipopolysaccharide group shows cell damage (red arrow), shrinkage of neurons (blue arrow), and basophilic neurons with core pyknosis (arrowhead) and D): treated group shows normal structure of the neurons. Notice few cell damage (red arrow), and shrinkage of neurons (blue arrow) (H and E, Scale bar 20 µm)

Histopathological investigation of brain sections of control and apigenin (0.78 mg /kg b. w.) showed the highly active nerve cells (neurons) that having huge nuclei with relatively pale-stained, the nuclear chromatin and prominent nuclei disappeared, the surrounding support cells (glial cells) having small nuclei with densely stained, condensed chromatin with no visible nucleoli and background substance (neuropil) are shown in the cortex (figure 2: A, B).

Microscopic examination of brain sections of LPS group showed shrinkage of neurons, cells damage, and basophilic neurons with core pyknosis (figure 2: C). While, in case of treated group, cerebrum showed normal structure of the neurons. Although, few cell damage and shrinkage of neurons were noticed (figure 2: D).

Wehr et al. [27] observed a marked decreased in serum PON-1 activity in neurodegenerative conditions as was found in our study which is possibly due to elevated levels of oxidative stress after LPS injection.
Our results are in agreement with Halliwell [28] who reported that, the increased of brain oxidative stress has been linked to the development of neurodegenerative diseases, thus, in this study, LPS injection significantly increased brain monoamines. In addition, accumulating evidence supports a role of inflammation in the pathogenesis of neurodegenerative disorders, such as Parkinson’s disease, Alzheimer’s disease, and schizophrenia, as well as depression [29].

Nonsteroidal hormones, such as noradrenaline, are able to cause oxidative DNA damage [30, 31]; the release of catecholamines in amounts exceeding physiological concentrations as was found in our study has been observed to exert cytotoxic effects in neuroblasts [31].

In this study, we used apigenin 7-O-β-glucoside extracted from dried powder of parsley seeds in order to attenuate lipopolysaccharide induced brain injury in experimental rat model.

We found that, apigenin supplementation significantly decreased oxidative stress in treated group which appeared in the reduction of MDA, nitric oxide and DNA damage as well as elevation of PON-1, these results may be related to the nature of apigenin; thus flavonoids were reported to scavenge free radicals and react with superoxide anion in various experimental systems [32]. Duthie and coworkers used the comet assay and observed the protective action of flavonoid against hydrogen peroxide-induced DNA damage in human lymphocytes [33]; the protection in that experiment was probably due to free radical-scavenging efficiency of the used flavonoid which may be associated with the presence of two hydroxyl groups in the B-ring of its molecule [34].

Concomitantly, the protection in our experiment may be due to free radical-scavenging efficiency of flavonoid apigenin, which may be associated with the presence of the hydroxyl groups in the B-ring of its molecule.
In conclusion, the current results suggest that apigenin protects neurons against oxidative stress-induced cell death via hydroxyl groups at B-ring which is critical for biological activities including the antioxidant and anti-inflammatory activity. The present study provides insights into the design of flavonoid with optimal neuroprotective activities.

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**REFERENCES**