



## Panax ginseng attenuates experimental brain injury by increasing brain-derived neurotrophic factor and inhibition of neuroinflammation

Jihan Hussein<sup>1\*</sup>, Eman Refaat<sup>1</sup>, Dalia Medhat<sup>1</sup>, Mona El-Bana<sup>1</sup>, Yasmin Abdel Latif<sup>1</sup>,  
Abdel Razik Farrag<sup>2</sup>, Naglaa Nazeef<sup>3</sup> and Mohamed Moify<sup>4</sup>

<sup>1</sup>Medical Biochemistry Department, National Research Centre, Doki, Giza, Egypt (Affiliation I D: 60014618)

<sup>2</sup>Pathology Department, National Research Centre, Doki, Giza, Egypt (Affiliation I D: 60014618)

<sup>3</sup>Phytochemistry Department, National Research Centre, Doki, Giza, Egypt (Affiliation I D: 60014618)

<sup>4</sup>Critical Care Center, El-Kasr El Einy

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### ABSTRACT

To investigate the protective effect of Panax Ginseng against lipopolysaccharide (LPS) induced brain injury in rat model. Rats were divided into four groups including control, ginseng, LPS and treated groups. Brain acetylcholinesterase, nitric oxide and brain derived neurotrophic factor (BDNF) were measured. Homocysteine was estimated by HPLC. Also, cyclooxygenases were determined by Immunohistochemistry in addition to histopathological investigations. Our results showed an elevation of both brain nitric oxide and serum homocysteine concomitant with a reduction of BDNF level, whereas Panax Ginseng significantly attenuated these data in treated group compared to LPS group. Histopathological and immunohistochemistry results confirmed these results. The important role of ginseng in attenuating brain injury may be related to the collection of ginsenosides that give ginseng a unique neuroprotective effect. Ginseng represents a promising neuroprotective therapy due to its anti-inflammatory properties.

**Key words:** homocysteine, cyclooxygenases, Nitric oxide, HPLC, acetylcholinesterase,

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### INTRODUCTION

Brain injury is a damage resulting from a mechanical force, such as rapid acceleration, impact, or penetration by a projectile [1]. Brain functions are impaired temporarily or permanently and the structural damage may be detectable with a current technology [2]. The role of inflammation in brain injury has also become better understood. Brain injury elicits a local inflammatory response involving the activation of astrocytes and microglia, local cytokine production, recruitment and infiltration of immuno-inflammatory cells. This inflammatory response has been shown to play a reparative role in response to the injury. However in the acute phase, this response may contribute to neuronal injury and cell death [3].

Lipopolysaccharide (LPS), the major component of the outer membrane of gram negative bacteria acts as endotoxin and elicits strong immune responses in experimental animals [4]. It is used as a model for studying the selective effects of inflammatory reaction on dopaminergic system and for studying brain dysfunction [5].

Inflammation reaction is started by cyclooxygenase-2 (COX-2) derived prostaglandins (PG) which promote inflammation by increasing vascular permeability and vasodilatation and by directing the synthesis and migration of pro-inflammatory cytokines into the site of inflammation [6]. The pro-inflammatory functions of COX-2 are linked primarily to exaggerated synthesis of prostaglandin 2 (PGE2) [7].

It was found that, LPS administration significantly decreased brain-derived neurotrophic factor (BDNF) which contributes to cognitive impairment [8]. Therefore, it may be possible to prevent LPS- induced cognitive dysfunction by increasing BDNF and inhibiting neuroinflammation.

Medicinal plants have been used for the treatment of various acute and chronic diseases [9]. Panax Ginseng is one of the unique herbs that include large number of ginsenosides. It is used in traditional medicine in several countries [10] and used in treatment of many diseases such as diabetes [11], renal dysfunction [12], and Alzheimer's disease [13]. Considering the potential effects of ginseng as anti inflammatory agent, we investigated whether administration of ginseng root had any protective effect against LPS - induced inflammation and reduction of BDNF in rat model and studied its ability to ameliorate brain injury .

## EXPERIMENTAL SECTION

### Materials

#### Chemicals

Lipopolysaccharide, homocysteine standard and all other chemicals (HPLC grade) were purchased from Sigma Aldrich Chemicals Company (St Louis, Missouri, USA).

#### Plant

Panax Ginseng root was purchased from a local market.

### Experimental animals

Forty male albino rats weighing  $150 \pm 10$  gram 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 a controlled temperature, under 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 animals' treatment followed the regulations of the ethical committee of the National Research Centre.

### Methods

#### Experimental design

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

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

Group II (ginseng): healthy rats, received ginseng root powder dissolved in ultrapure water (22mg /kg body weight / day) orally for ten days.

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

Group IV (treated group): healthy rats, received ginseng extract (22mg /kg body weight /day) orally for ten days followed by i.p. injection of lipopolysaccharide (LPS; 100 mg/kg body weight once).

After four hours of LPS injection, blood was withdrawn from the retro-orbital venous plexus of the eye using heparinized capillary tubes and collected in dry clean tubes for serum separation.

Brain was removed quickly, washed with cold saline solution (0.9% NaCl) and divided into two parts; the first part was homogenized and prepared for biochemical estimations and the 2<sup>nd</sup> part was prepared for histopathological and immunohistochemical analysis.

#### Preparation of tissue homogenate

Brain tissues were homogenized in phosphate buffer (pH 7.4), centrifuged at 4000 rpm for 15 minutes at 4°C using cooling centrifuge (Laborzentrifugen, 2K15, Sigma, Germany) and the supernatant was removed for chemical parameters estimation [14].

### Biochemical analysis

#### Determination of brain nitric oxide level

Nitric oxide measured as nitrite was determined by using Griess reagent, according to the previous method [15] where nitrite, the stable end product of nitric oxide radical, is mostly used as indicator for the production of nitric oxide.

**Determination of brain acetylcholinesterase**

The cholinesterase present in the sample catalyzes the hydrolysis of the butyrylthiocholine and forming butyrate and thiocholine. The later reduces the exacyanoferrate (III) to exacyanoferrate (II). The decrease of absorbance per unit time at 405 nm is correlated with the activity of cholinesterase in the sample [16].

**Brain-derived neurotrophic factor (BDNF)**

Brain BDNF level was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Neobioscience Technology Company) according to the manufacturer's protocols.

**Determination of serum Homocysteine (Hcy)**

Homocysteine was estimated as described previously [17,18] using high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (pump, G131A model) and UV detector.

**Sample extraction**

Briefly, 200  $\mu$ l serum was added to 16  $\mu$ l of 1.2 mol trichloro acetic acid (TCA), mixed well and incubated for 30 min. in ice to precipitate protein. After centrifugation for 20 min. at 4000 rpm and 4°C, supernatants were filtered through hydrophilic PVDF 0.45  $\mu$ m filter.

**HPLC condition**

From the filtered solution, 30  $\mu$ l were injected onto HPLC; separation was achieved on reversed phase column (C18, 250, 0.46 cm i.d. 5  $\mu$ m). The mobile phase consisted of 40 mmol sodium phosphate monobasic monohydrate; 8 mmol heptanesulfonic acid and 18% (v/v) methanol, the mobile phase was adjusted to pH 3.1 by addition of phosphoric acid and filtered through 0.45- $\mu$ m membrane filter and delivered at a flow rate of 1 ml/min at 40°C. UV detection was performed at 260 nm. Serial dilutions of standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus their corresponding concentrations. Samples concentrations were obtained from the standard curve.

**Histopathological investigations**

The brain was fixed in formalin for 24 hours. The specimens were washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax (melting point 55-60 °C). Sections of 6 mm thickness were prepared and stained with Haematoxylin and eosin [19]. Paraffin sections were stained in Harris's haematoxylin for 5 minutes and washed in running water 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, 5 $\mu$ m-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 anticyclooxygenase-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  $\pm$  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 when  $p \leq 0.05$ .

**RESULTS**

Our results indicated that, LPS injection in a dose of 100 mg/kg body weights significantly increased both brain nitric oxide and serum homocysteine levels compared to control group. In contrast; it decreased brain paraoxonase 1, cholinesterase and BDNF ( $p \leq 0.5$ ) as was found in Figs 1-5.

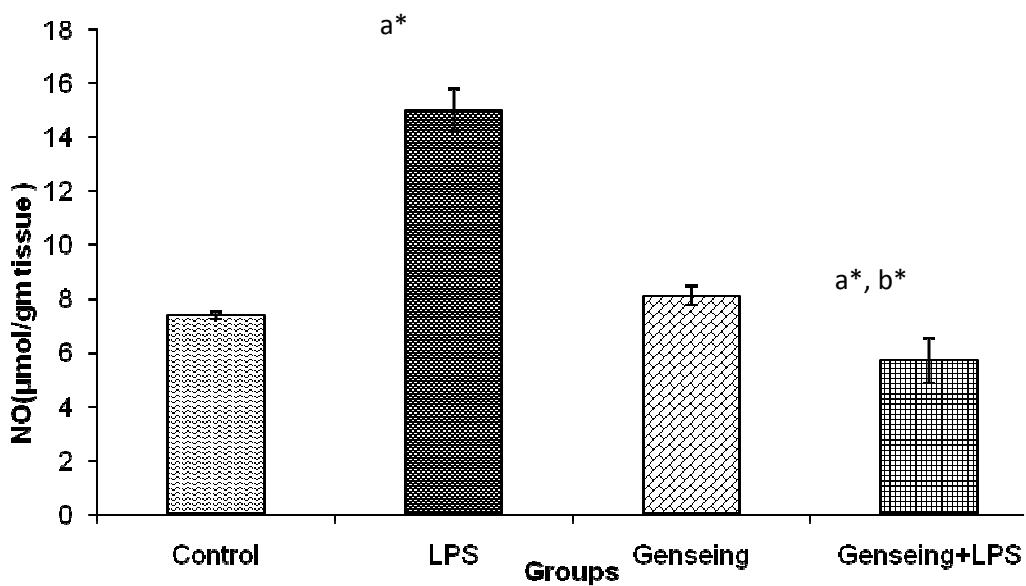


Fig 1. Brain nitric oxide level in different studied groups

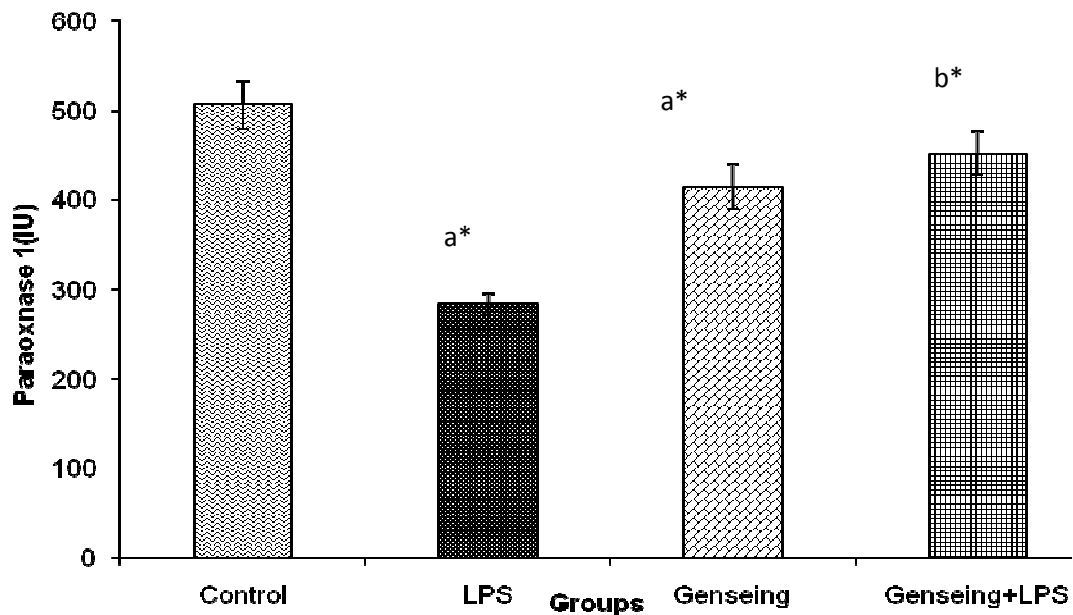


Fig 2. Brain paraoxonase 1 activity in different studied groups

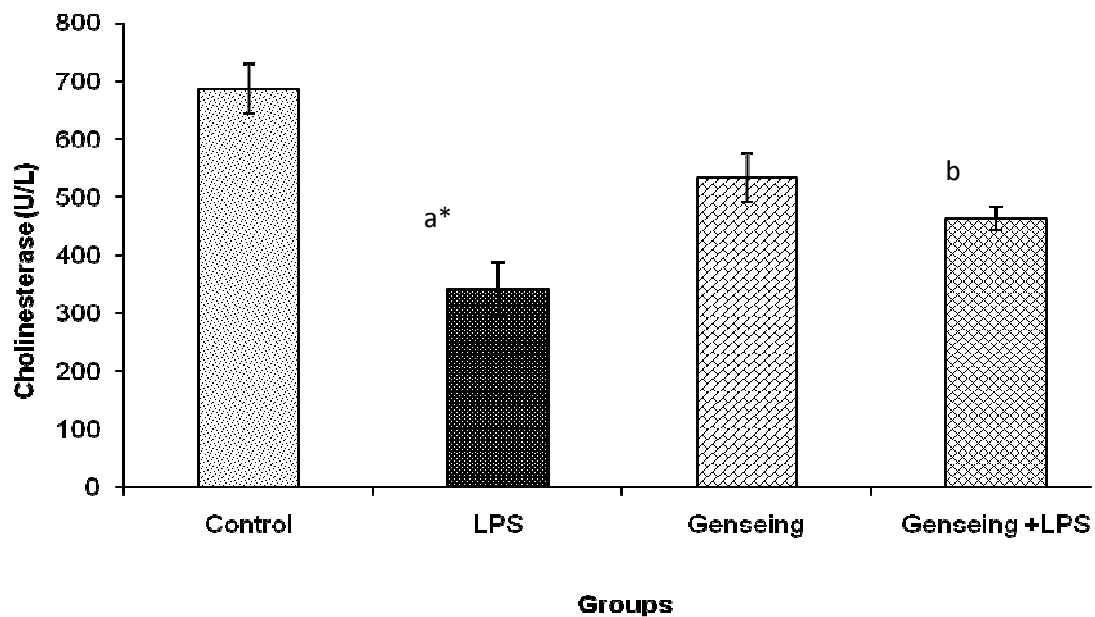


Fig 3 : Brain cholinestrace in different studied groups

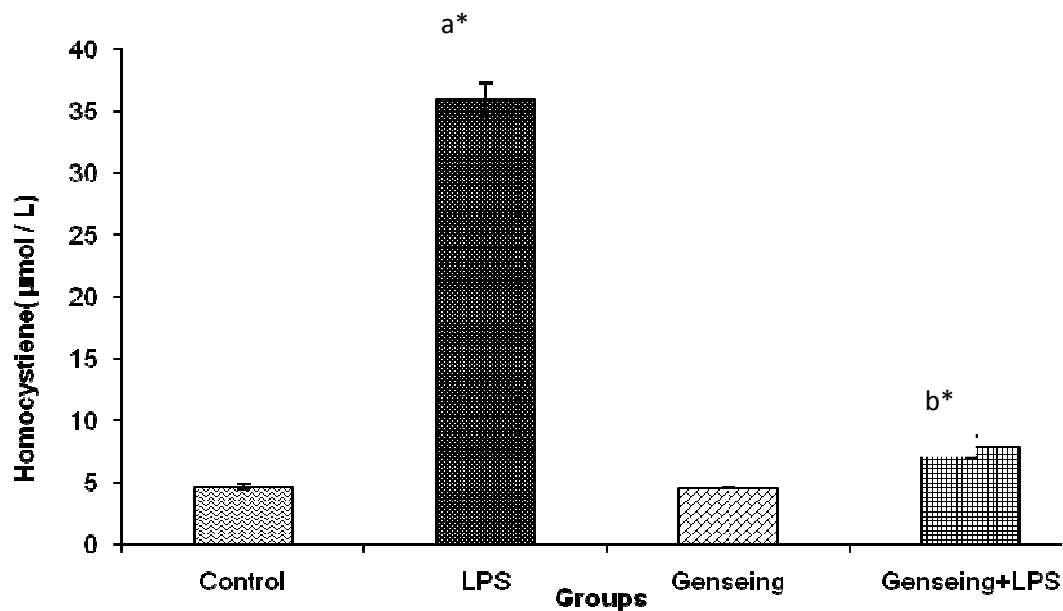


Fig 4. Serum homocysteine levels in different studied groups

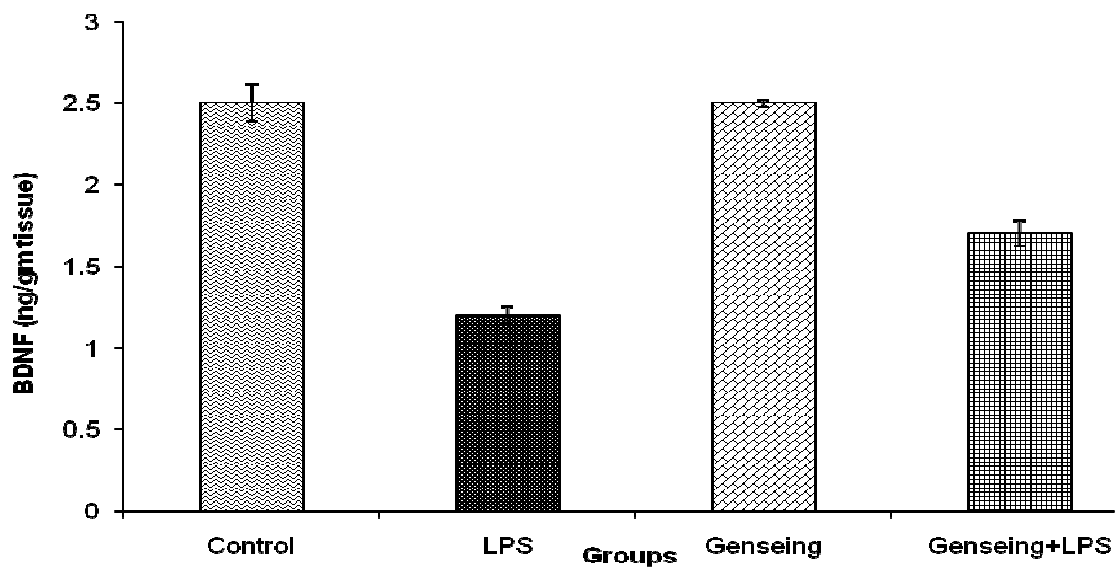


Fig 5. Brain BDNF in different studied groups

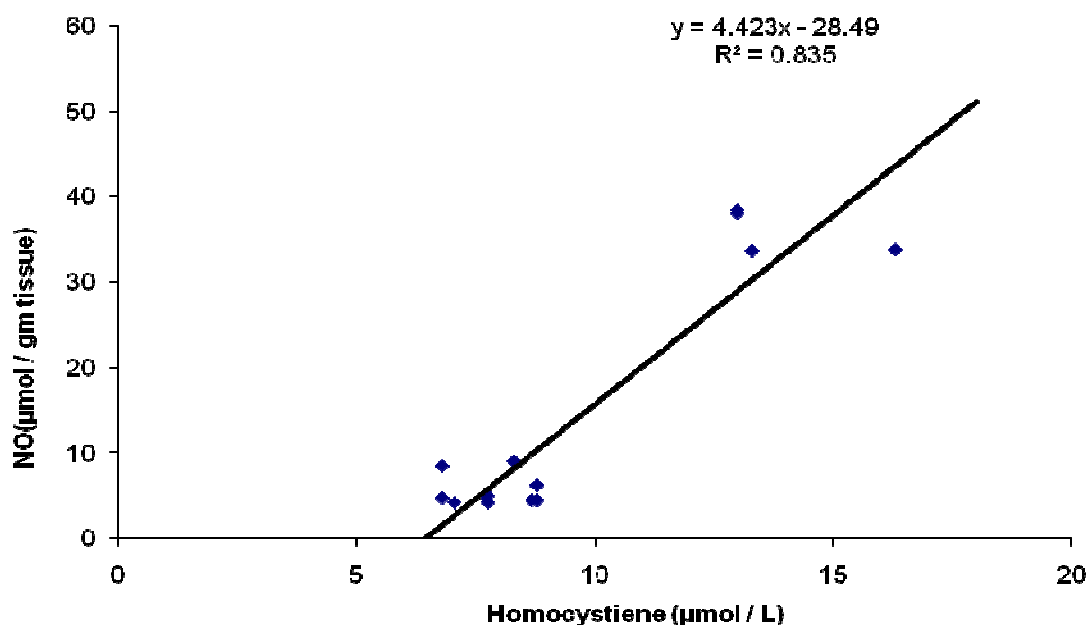
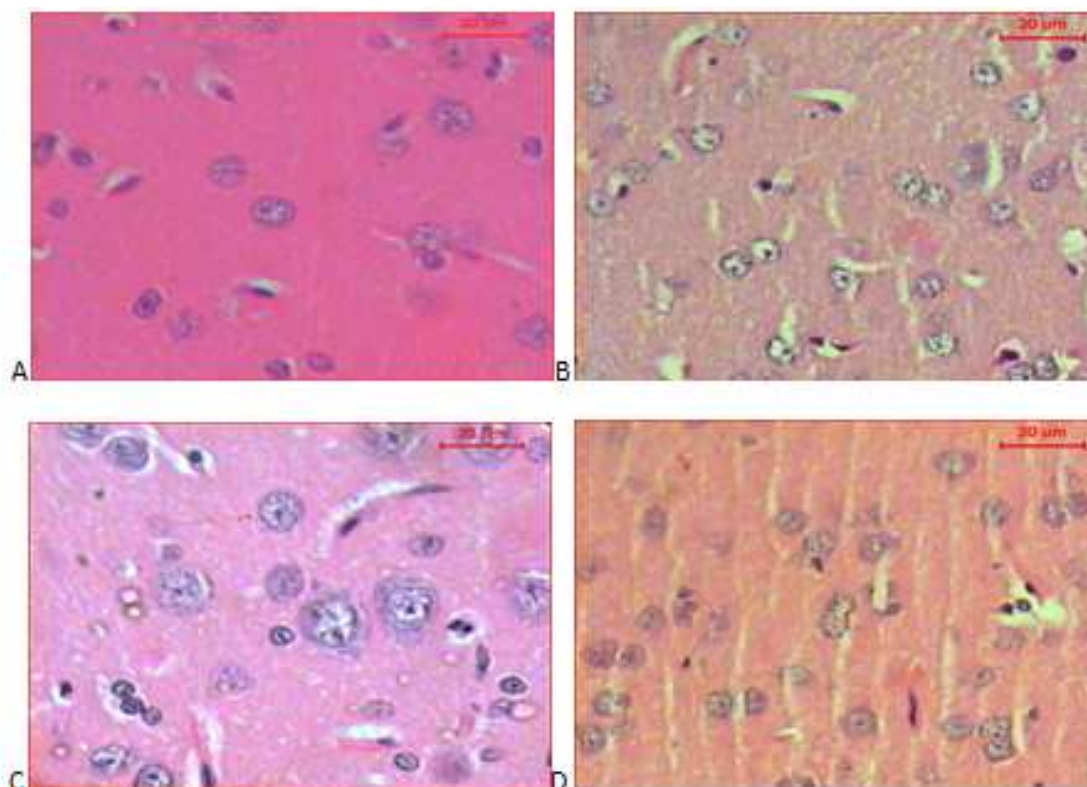
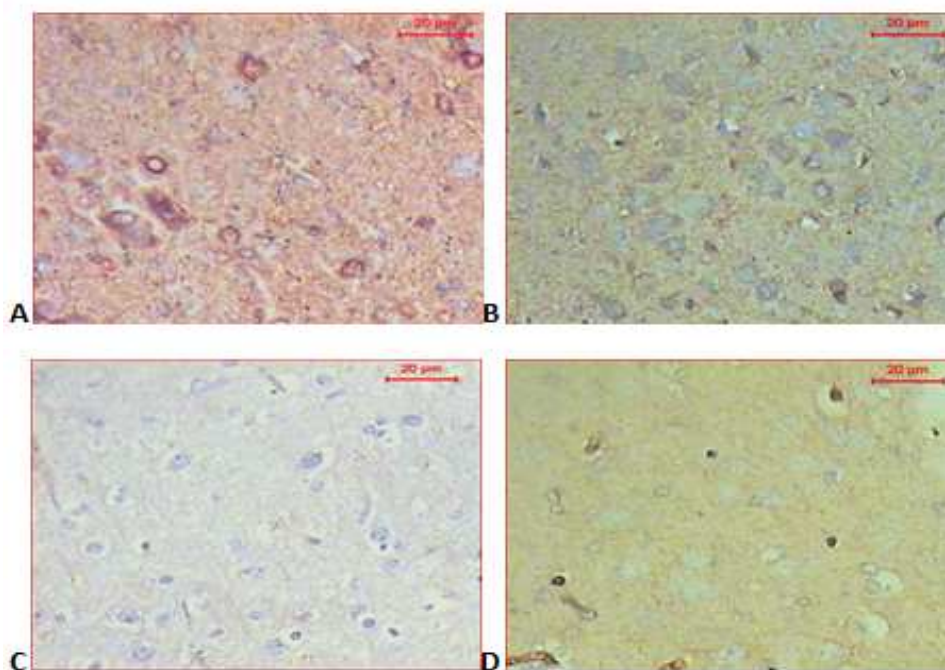


Fig 6. Positive correlation between homocysteine and NO in different studied groups



**Fig 7. Micrographs of sections in brain cerebrum**

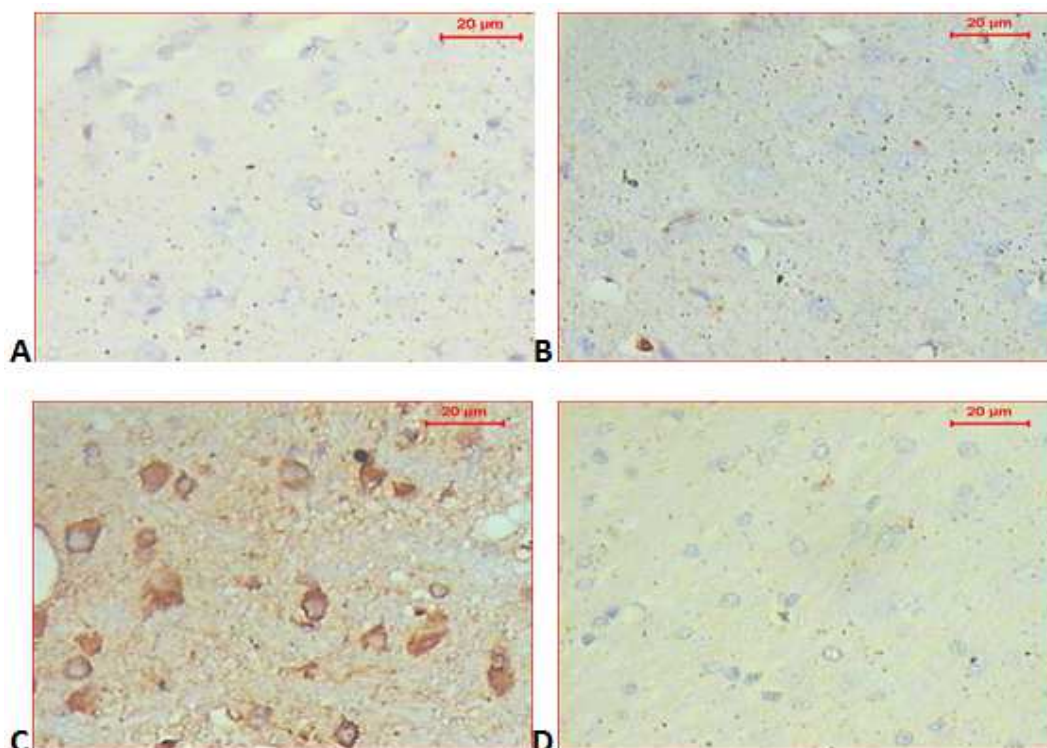
(A) control shows the highly active nerve cells that having huge nuclei with relatively pale-stained, the nuclear chromatin and prominent nucleoli disappeared, the surrounding support cells having small nuclei with densely stained, condensed chromatin with no visible nucleoli and background substance are shown in the cortex, (B) rat given Demso shows the normal structure of cerebrum, (C) healthy rat given oral dose of ginseng (22mg /kg body weight / day ) for ten days shows the normal structure of cerebrum, (D) healthy rat received a vehicle for ten days followed by intraperitoneal (i.p.) injection of lipopolysaccharide (100 mg/kg b. w.) shows cell damage (red arrow), shrinkage of neurons (blue arrow), and basophilic neurons with core pyknosis arrowhead (H and E, Scale bar 20  $\mu$ m).



**Fig 8. Micrographs of sections in brain cerebrum**

( A) control,(B) healthy rat given oral dose of ginseng (22mg /kg body weight / day ) for ten days shows positive reaction of Cox 1 as indicated by brown color, (C) healthy rat received a vehicle for ten days followed by intraperitoneal (i.p.) injection of lipopolysaccharide (100 mg/kg b. w.) shows negative reaction of Cox 1, (D) healthy rats, received apigenin for ten days followed by intraperitoneal (i.p.) injection of lipopolysaccharide shows positive reaction (brown color) (Cox 1 immunostaining, Scale bar 20  $\mu$ m).

A positive correlation was observed in our study between NO and homocysteine (Fig 6). The biochemical results were confirmed by the pathological and immunohistochemical results (Figs 7-9).



**Fig 9. Micrographs of sections in brain cerebrum**

(A) control, (B) healthy rat given oral dose of ginseng (22mg/kg body weight / day ) for ten days shows negative reaction of Cox 2, (C) healthy rat received a vehicle for ten days followed by intraperitoneal (i.p.) injection of lipopolysaccharide (100 mg/kg b. w.) shows positive reaction of Cox 2 that appear as brown color, (D) healthy rats, received apigenin for ten days followed by intraperitoneal (i.p.) injection of lipopolysaccharide shows little amount of brown color (Cox 2 immunostaining, Scale bar 20 µm).

## DISCUSSION

LPS-induced inflammatory reactions by non-enzymatically oxidized phospholipids (OxPLs). The non-enzymatically oxidized phospholipids, the major component of oxidized low density lipoprotein is characterized by its ability to induce synthesis of cytokines and chemokines [20].

Previous studies proved that LPS, cytokines [21] and stress [22] lead to elevation of NO levels by iNOS activation. In this study, we measured nitrate/nitrite levels, which are known as the end products of NO [23] and established a statistically significant increase in LPS group compared to control group. The elevation of nitric oxide level in this study was considered as successful demonstration of LPS- induced inflammation and endotoxemia in rat model.

Our data showed a relationship between NO and homocysteine. It has been reported that homocysteine stimulates NO production by stimulating eNOS [24]. Thus, it stimulates iNOS expression in macrophages [25].

In addition, Hcy might generate oxygen species that attack fatty acids in the cell membrane resulting in liberation of the powerful arachidonic acid-derived mediators of inflammation to produce the prothrombotic agent thromboxane A<sub>2</sub> and pro-inflammatory cytokines, such as IL-1 $\beta$  [26].

The presence of COX 2 and the absence of COX1 in brain tissue of LPS group in this study confirmed this suggestion.

This inflammation interferes with BDNF gene expression and function, the reduction of BDNF may be an important factor involved in the development of brain dysfunction; however, treatment with *Panax Ginseng* restored this level. Ginseng is unique, in comparison to other neuroprotective agents because it is not one single compound but a collection of compounds, called ginsenosides. Thus, there are more than sixty known ginsenosides [27], which produce different effects through several sites of action [28].



In this study, Ginseng supplementation significantly increased Brain paraoxonase 1 activity (the anti oxidative stress marker) in treated group compared to LPS group.

Ginsenoside Rg1 was found to suppress oxidative stress and block activation of JNK signaling [29, 30], in addition to activation of insulin-like growth factor receptors to protect dopaminergic neurons [31]. Moreover, the ginsenoside Re was reported to regulate nitric oxide levels [32] as was found in our study.

In the current study, ginseng effectively attenuated the elevation of homocysteine and the presence of COX-2, the inflammatory marker in the treated group beside the appearance of COX-1.

This anti-inflammatory action of ginseng which was shown in the current study is linked to Rg3- and Re-mediate upregulation of cytokines along with iNOS and COX-2[33, 34]. Ginsenosides Rg1 and Rb1 have also been found to induce the expression of growth factors like brain-derived neurotrophic factor (BDNF) [35,36] and to activate its signaling pathways [37] Thus, our results showed a significant increase in brain BDNF level in treated group compared to LPS group.

### CONCLUSION

*Panax ginseng* provided near complete protection against brain injury induced by LPS. Our data suggest that *Panax Ginseng* represents a promising and effective neuroprotective therapy due to its anti-inflammatory and anti-oxidant properties in addition to its role in maintaining brain-derived neurotrophic factor.

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