Estrogen Enhances the Inhibitory Effect of Iron on Microglial Nitric Oxide Production

Poonlarp Cheepsunthorn PhD*,
Nootchanat Mairaue MSc*, Krongkan Nasee BSc*

** Department of Anatomy, Faculty of Medicine, Chulalongkorn University*

Abnormal iron accumulation has been consistently reported in specific brain regions of many neurodegenerative diseases. At cellular level, iron is unusually observed in microglia, immune effector cell of the brain. Most evidence has provided that upon activation, microglia produces neurotoxins and different kinds of inflammatory mediators. Therefore, it is believed that activated microglia is actively involved in neurodegenerative process. Using a rat microglial cell line (HAPI), the present study was designed to address the role of iron for immune function of microglia, in particular, the production of Nitric Oxide (NO) in the presence or absence of estrogen, a potential neuroprotective agent. The present results demonstrated that exposure of microglia to iron significantly decreased lipopolysaccaride-induced NO production, as determined by nitrite accumulation in the cell culture medium, and such effect of iron was potentiated by increasing concentration of estrogen. Transcript analysis revealed that estrogen, but not iron, decreased the expression of inducible Nitric Oxide Synthase (iNOS). These results demonstrate that estrogen enhances the inhibitory effect of iron on microglial NO production by decreasing mRNA expression of iNOS and also suggest that iron sequestration by microglia under neuropathological conditions could be a protective mechanism against NO-induced neurotoxicity.

Keywords: *Glia, Iron, iNOS, Neuroinflammation*

J Med Assoc Thai 2006; 89 (6): 840-5
Full text. e-Journal: *http://www.medassocthai.org/journal*

Deposition of excess iron in the brain and activation of microglia have been implicated as major causes of progressive cell loss in many neurodegenerative disorders. In the brains of patients with Alzheimer's disease, iron level in the amyloid plaque is increased approximately three-times higher than that of control neuropil[1]. In Parkinson's disease and stroke, the increase in brain iron content is specific for dopaminergic neuron-containing substantia nigra and the perimeter of ischemic lesion, respectively[2-4]. The toxicity of iron, even in a bound form, is due to its catalytic ability to generate highly toxic hydroxyl radical ($^{\cdot}OH$) from superoxide and hydrogen peroxide released in the normal process of mitochondrial energy respiration and in the process of microglial activation[5,6]. Furthermore, superoxide anion can readily react with excessive amounts of Nitric Oxide (NO) produced by inducible Nitric Oxide Synthase (iNOS), a high output enzyme up-regulated following microglial activation, to form reactive nitrogen intermediate or peroxynitrite ($ONOO^-$)[7,8]. Together, $^{\cdot}OH$ and $ONOO^-$ can cause a direct or delayed damage to both neurons and glia by oxidatively modifying or cross-linking cellular proteins, lipid membrane and deoxy-nucleic acids[9].

Several lines of evidence have shown that secretory products from activated microglia including pro-inflammatory mediators contribute to neural tissue damage, while factors that interfere with the process of microglial activation, such as minocycline, p38 MAPK inhibitor and estrogen limit the damage[10-15]. Although, microglia, like other cell types, requires well-defined amounts of iron for basic cellular functions, a recent study demonstrates that cellular iron accumulation, as a result of elevated levels of extracellular iron, can modify gene expression profiles in microglia challenged with

Correspondence to : Cheepsunthorn P, Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Rama IV Rd, Bangkok 10330, Thailand. E-mail: Poonlarp.C@chula.ac.th

inflammatory stimulus[16]. A more detailed knowledge of the interaction between microglia and these candidate neuroprotective agents under pathological-related, iron-rich environment could, therefore, lead to more effective treatments for neurodegenerative diseases aimed to target microglial activation. The present study aimed to examine the effects of iron on LPS-induced NO production in microglial cells in the presence or absence of estrogen by using an established rat microglial cell line[17], HAPI cells.

Material and Method

Cell culture and Cell treatment

Immortalized rat microglia line HAPI[17] were maintained in Dulbecco's modified eagle's medium (DMEM) containing 5% fetal bovine serum (FBS; HyClone), L-glutamine (20 mM) and penicillin-streptomycin solution (10ml/l) at 37^{0}C under humidified 5% CO_2. Cells were seeded in 24-well plates at the density of 5x10^4/well. Approximately 24 hrs later, the medium was removed, replaced with freshly prepared serum-free medium containing lipopolysaccharide (LPS; 1 μg/ml), or ferric ammonium citrate [FAC; 50 μg/ml as previously described[16,17]], or 17β-estradiol as indicated in each experiment, and incubated for 24 h (for NO determination) or 18 h (for determination of iNOS transcript levels). All tissue culture reagents were purchased from Sigma, unless otherwise stated.

Nitrite assay

The generation of NO was determined by measuring nitrite accumulation in the cell culture supernatants using Griess reagent (Invitrogen). Briefly, 150 μl/well of the nitrite-containing sample were placed in a 96-well plate, mixed with 20 μl of Griess reagent and 130 μl of deionized water and incubated for 30 min at room temperature. Optical density was read at 540 nm and compared with a standard curve generated in each experiment from known concentration of nitrites.

RT-PCR analysis

After 18 h treatment, total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. A volume of 1 μg total RNA from each sample was subjected to reverse transcription (RT) using 1st strand cDNA synthesis kit for RT-PCR (Avian Myeloblatosis Virus (AMV); Roche) in a final volume of 20 μl. The RT reaction was performed at 42^{0}C for 1 h, the enzyme was inactivated at 99^{0}C for 5 min, and cDNA samples were stored at -20^{0}C in 100 μl of final volume.

Five microliters of each cDNA sample was used as template for subsequent PCR reaction in 50 μl of final volume in the presence of Taq DNA polymerase (Promega) and primers specific for rat inducible nitric oxide synthase (iNOS); forward (5'-atcccgaaacgct acacttcc-3'), reverse (5'-ggcgaagaacaatccacaactc-3') resulting in 311 bp products or for glyseraldehyde-3-phosphate dehydrogenase (GAPDH); forward (5'-aagctcactgg catggccttcc-3'), reverse (5'-ttggaggccatgt aggccatgag-3') resulting in 300 bp products. The PCR reactions were performed on Perkin-Elmer Thermal Cycle as follows: 95^{0}C for 5 min followed by 35 cycles at 94^{0}C for 1 min, 58^{0}C (61^{0}C for GAPDH) for 1 min, and 72^{0}C for 2 min. The amplification products were analyzed on 1.2% agarose gel. Band intensity was then quantified by densitometry.

Statistical analysis

Data were presented as mean $\pm$ SEM of three independent experiments, in which triplicate samples were performed. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni test to determine the significance of difference, assigned to the level of $p < 0.05$.

Results

Iron exposure significantly decreases LPS-induced microglial NO production

To address the role of iron for NO production in activated microglia, the authors challenged HAPI cells with 1 μg/ml LPS for 24 hrs in the presence or absence of 50 μg/ml FAC followed by an evaluation of nitrite (NO_2^-) levels in the cell culture media. As shown in Fig. 1, LPS induced approximately 3-fold increases in NO_2^- level, which was counteracted by simultaneous exposure to iron.

Estrogen enhances the inhibitory effect of iron on LPS-induced microglial NO production

Previous immunohistological study has shown that microglia express estrogen receptors[18], suggesting microglial activation could be modified by estrogen. The authors then asked whether the effect of iron on microglial NO production could be modified by estrogen. In this set of experiments, HAPI cells were cultured for 24 h under different treatment conditions as indicated and NO_2^- accumulation in the cell culture media was determined. As shown in Fig. 2, addition of iron had no effect on baseline NO_2^-, but it caused a 30% decrease in NO_2^- level in LPS-treated group. 17β-estradiol treatment decreased NO_2^-accumulation in a

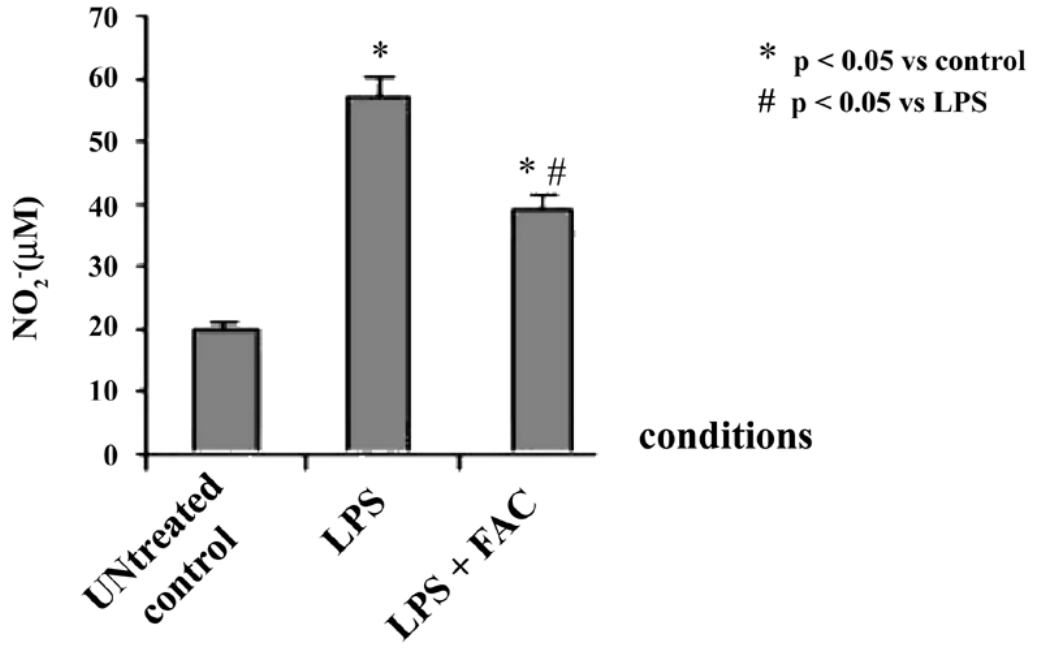

Fig. 1 HAPI, rat microglial cells, were treated with lipopolysaccharide (LPS; 1 mg/ml) in the presence or absence of ferric ammonium citrate (FAC; 50 mg/ml) for 24 h. Then, the concentration of NO_2^- in the cell culture media was determined by Griess reaction. Results were representative of three independent experiments. As indicated, * $p < 0.05$, as compared with the control and # $p < 0.05$, as compared with LPS treatment

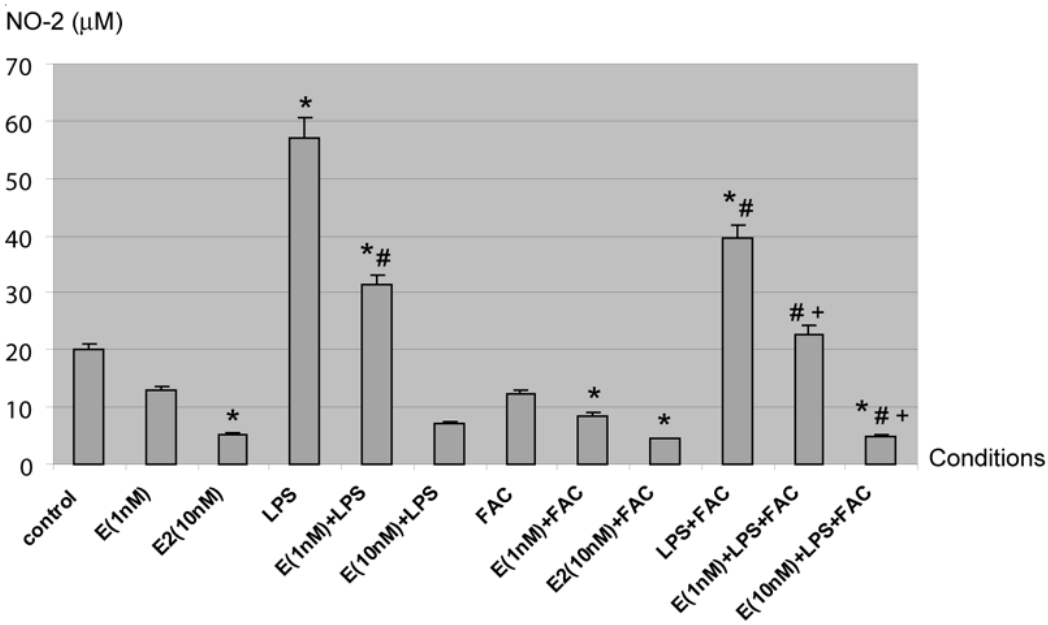

Fig. 2 HAPI cells were treated with lipopolysaccharide (LPS; 1 μg/ml) in the presence or absence of ferric ammonium citrate (FAC; 50 mg/ml) or 17β-estradiol (1 nM or 10 nM) for 24 h. Then, the concentration of NO_2^- in the cell culture media was determined by Griess reaction. Results were representative of three independent experiments. As indicated in the graphs, * $p < 0.05$, compared with the control; # $p < 0.05$, compared with LPS treatment alone; and + $p < 0.05$, compared with LPS + FAC treatment, were calculated by ANOVA followed by Bonferroni test

dose-dependent manner. Interestingly, when the cells were treated with LPS and iron in the presence of 1 nM 17β-estradiol, NO_2^- levels were reduced to 37% compared with LPS and 55% compared with LPS + iron. When a 10 nM concentration of 17β-estradiol was used, a similar, but greater, effect was obtained (8%; compared with LPS and 12%; compared with LPS + iron). These results suggest a regulatory role of estrogen and iron in microglial NO production.

Estrogen, but not iron, decreases LPS-induced microglial iNOS expression

To investigate whether the inhibitory effects of iron and estrogen on microglial NO production occur at the transcript level, we examined the mRNA expression of iNOS in HAPI cells at 18 hrs under conditions indicated in Fig. 3. Treatment with 17β-estradiol decreased iNOS mRNA in untreated and LPS-treated cells in a dose-dependent manner, consistent with NO_2^- results obtained at 24 h. Exposure to iron did not affect iNOS transcripts either in untreated or in LPS-treated cells, suggesting a non-genomic effect of iron on NO production. A concentration of 10 nM 17β-estradiol, but not 1 nM, significantly reduced iNOS transcript levels (65% as compared with LPS + iron). These results demonstrate that estrogen enhances the inhibitory effect of iron on microglial NO production by down-regulating iNOS mRNA expression.

Discussion

A growing body of *in vitro* studies suggest that a delayed death of cortical and mesencephalic (dopaminergic) neurons following primary insult is mediated by microglia-derived NO, which appear to be attenuated by NOS inhibitors[19-22]. However, under pathological setting in the brains of patients with Alzheimer's and Parkinson's diseases, there is an elevated level of iron in the degenerated areas where iron is abnormally accumulated in microglia[23,24]. Although, little is known about the role of iron for microglial function, the authors have previously shown that addition of iron to cultures of LPS-activated microglia alters their gene expression patterns and leads to intracellular iron accumulation, resembling iron-

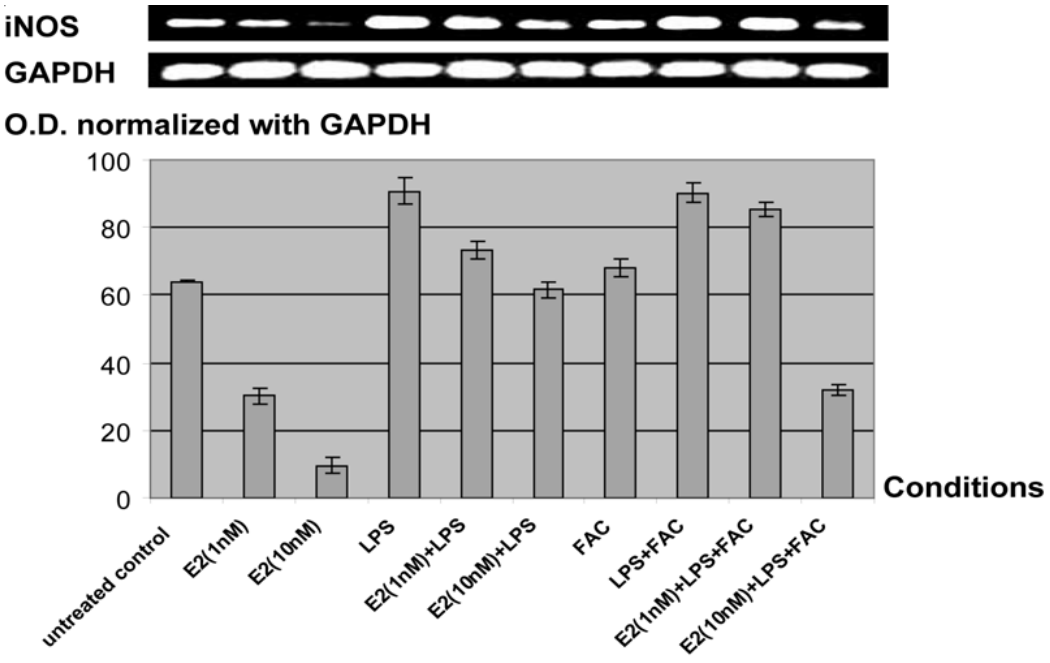

Fig. 3 HAPI cells were treated with lipopolysaccharide (LPS) in the presence or absence of ferric ammonium citrate (FAC) or 17β-estradiol (1 nM or 10 nM) for 18 h. Total RNA was extracted and mRNAs for inducible nitric oxide synthase (iNOS) and glyseraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by RT-PCR assay. A representative of amplification products separated by agarose gel eletrophoresis was shown. The densitometric evaluation of the bands obtained from two-independent experiments were reported as mean ± SEM. As indicated in the graphs, * $p < 0.05$, as compared with the control values; # $p < 0.05$, compared with LPS treatment alone; and + $p < 0.05$, compared with LPS + FAC treatment, were calculated by ANOVA followed by Bonferroni test

laden phenotype of activated microglia in many neurodegenerative diseases[16,17]. In the present study, the authors employed the described *in vitro* model to understand better the involvement of iron in microglial NO production. The results showed that NO production from LPS-activated microglia in the presence of iron was significantly reduced without affecting iNOS mRNA expression. These results correspond to a previous report, in which cultures of hepatocytes exposed to LPS and γ-interferon in the presence of iron showed a decrease of NO and an increase of NO bound to iron-containing molecules (nitrosyl-iron complexes)[25]. Thus, the ability of iron to reduce microglial NO may protect neurons from a delayed cell death mediated by NO.

The mechanism by which estrogen enhances the inhibitory effect of iron on NO production from activated microglia appear to be iron-independent and occur via the down-regulation of iNOS mRNA. This is consistant with previous studies that estrogen was capable of decreasing iNOS expression[26]. Although the authors have not directly shown the presence of estrogen receptors α or β in the present study, the results suggest that the HAPI cells express functional receptors for estrogen. Thus, the HAPI cells may provide an alternative cell culture model for investigating effects of estrogen on inflammatory component of microglia-mediated neurodegeneration. Studies are in progress to define better the effects of estrogen on microglial function under diseased-oriented and iron-rich environment.

In conclusion, the authors propose that iron-laden activated microglia in neurodegenerative diseases could play a role in brain inherited defensive mechanisms against NO-induced neurotoxicity.

Acknowledgements

This work was supported by the Molecular Research Fund, Faculty of Medicine, Chulalongkorn University to P.C. and by Graduate School Chulalongkorn University to K.N.

References

1. Aisen P, Listowsky I. Iron transport and storage proteins. Annu Rev Biochem 1980; 49: 357-93.
2. Sofic E, Paulus W, Jellinger K, Riederer P, Youdim MB. Selective increase of iron in substantia nigra zona compacta of parkinsonian brains. J Neurochem 1991; 56: 978-82.
3. Jenner P, Olanow CW. Understanding cell death in Parkinson's disease. Ann Neurol 1998; 44: S72-84.
4. Panahian N, Huang T, Maines MD. Enhanced neuronal expression of the oxidoreductase biliverdin reductase after permanent focal cerebral ischemia. Brain Res 1999; 850:1-13.
5. Halliwell B, Gutteridge JM. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. FEBS Lett 1992; 307:108-12.
6. Colton CA, Snell J, Chernyshev O, Gilbert DL. Induction of superoxide anion and nitricoxide production in cultured microglia. Ann N Y Acad Sci 1994; 738: 54-63.
7. Boje KM, Arora PK. Microglial-produced nitric oxide and reactive nitrogen oxidesmediate neuronal cell death. Brain Res 1992; 587:250-6.
8. Egger T, Hammer A, Wintersperger A, Goti D, Malle E, Sattler W. Modulation of microglial superoxide production by alpha-tocopherol in vitro: attenuation of p67 (phox) translocation by a protein phosphatase-dependent pathway. J Neurochem 2001; 79: 1169-82.
9. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem 2005; 12: 1161-208.
10. Fan LW, Pang Y, Lin S, Tien LT, Ma T, Rhodes PG, et al. Minocycline reduces lipopolysaccharide-induced neurological dysfunction and brain injury in the neonatal rat. J Neurosci Res 2005; 82: 71-82.
11. Fan LW, Pang Y, Lin S, Rhodes PG, Cai Z. Minocycline attenuates lipopolysaccharide-induced white matter injury in the neonatal rat brain. Neuroscience 2005; 133: 159-68.
12. Krady JK, Basu A, Allen CM, Xu Y, LaNoue KF, Gardner TW, et al. Mino-cycline reduces proinflammatory cytokine expression, microglial activation and caspase-3 activation in a rodent model of diabetic retinopathy. Diabetes 2005; 54: 1559-65.
13. Piao CS, Kim JB, Han PL, Lee JK.Administration of the p38 MAPK inhibitor SB203580 affords brain protection with a wide therapeutic window against focal ischemic insult. J Neurosci Res 2003; 73: 537-44.
14. Hurn PD, Macrae IM. Estrogen as a neuroprotectant in stroke. J Cereb Blood Flow Metab 2000; 20: 631-52.
15. Liu X, Fan XL, Zhao Y, Luo GR, Li XP, Li R, et al. Estrogen provides neuro-protection against activated microglia-induced dopaminergic neuronal injury through both estrogen receptor-alpha and estrogen receptor-beta in microglia. J Neurosci Res 2005; 81: 653-65.

16. Connor JR, Cheepsunthorn P, Reid J, Radov L. Iron mediates gene expression in microglia. J Neurochem 2002; 81: 52S.
17. Cheepsunthorn P, Radov L, Menzies S, Reid J, Connor JR. Characterization of a novel brain-derived microglial cell line isolated from neonatal rat brain. Glia 2001; 35: 53-62.
18. Garcia-Ovejero D, Veiga S, Garcia-Segura LM, Doncarlos LL. Glial expression of estrogen and androgen receptors after rat brain injury. J Comp Neurol 2002; 450: 256-71.
19. Ryu JK, Shin WH, Kim J, Joe EH, Lee YB, Cho KG, et al. Trisialoganglioside GT1b induces in vivo degeneration of nigral dopaminergicneurons: role of microglia. Glia 2002; 38: 15-23.
20. Liu B, Gao HM, Wang JY, Jeohn GH, Cooper CL, Hong JS. Role of nitric oxide in inflammation-mediated neurodegeneration. Ann N Y Acad Sci 2002; 962: 318-31.
21. Abe K, Abe Y, Saito H. Agmatine suppresses nitric oxide production in microglia. Brain Res 2000; 872: 141-8.
22. Dawson VL, Brahmbhatt HP, Mong JA, Dawson TM. Expression of inducible nitric oxide synthase causes delayed neurotoxicity in primary mixed neuronal-glial cortical cultures. Neuropharmacology 1994; 33: 1425-30.
23. Kaneko Y, Kitamoto T, Tateishi J, Yamaguchi K. Ferritin immunohistochemistry as a marker for microglia. Acta Neuropathol 1989; 79: 129-36.
24. Jellinger K, Paulus W, Grundke-Iqbal I, Riederer P, Youdim MB. Brain iron and ferritin in Parkinson's and Alzheimer's diseases. J Neural Transm Park Dis Dement Sect 1990; 2: 327-40.
25. Sergent O, Griffon B, Morel I, Chevanne M, Dubos MP, Cillard P, et al. Effect of nitric oxide on iron-mediated oxidative stress in primary rat hepatocyte culture. Hepatology 1997; 25: 122-7
26. Vegeto E, Pollio G, Ciana P, Maggi A. Estrogen blocks inducible nitric oxide synthase accumulation in LPS-activated microglia cells. Exp Gerontol 2000; 35: 1309-16.

เอสโตรเจนมีฤทธิ์เสริมผลของธาตุเหล็กต่อการยับยั้งการสร้างไนตริกออกไซด์ในไมโครเกลีย

พูลลาภ ชีพสุนทร, นุชนาถ ไหมหรือ, กรองกาญจน์ นาสี

การสะสมของธาตุเหล็กอย่างผิดปกติในโรคทางระบบประสาทหลายชนิด มีความสัมพันธ์กับการเสื่อมของเซลล์ประสาท การศึกษาทางพยาธิวิทยาในระดับเซลล์พบว่าธาตุเหล็กจะสะสมอยู่มากในไมโครเกลีย ซึ่งถือเป็นเซลล์ภูมิคุ้มกันของระบบประสาทที่เมื่อถูกกระตุ้นสามารถหลั่งสารหลายชนิดที่เป็นอันตรายต่อเซลล์ประสาทได้ เพื่อตรวจสอบผลของธาตุเหล็กต่อการสร้างไนตริกออกไซด์จากไมโครเกลียในภาวะที่ถูกกระตุ้นขณะที่มีและไม่มีฮอร์โมนเอสโตรเจน ซึ่งเป็นสารที่ออกฤทธิ์ปกป้องเซลล์ประสาท ผลการทดลองจากการใช้ไมโครเกลียที่ได้มาจากสมองของหนู (HAPI เซลล์) แสดงให้เห็นว่าธาตุเหล็กสามารถลดการสร้างไนตริกออกไซด์จากไมโครเกลียที่ถูกกระตุ้นด้วย ไลโปโปลิแซคคาไรด์อย่างมีนัยสำคัญทางสถิติโดยดูได้จากปริมาณไนไตรต์ในอาหารเลี้ยงเซลล์ ทั้งนี้ยังพบว่า เอสโตรเจนสามารถเสริมผลของธาตุเหล็กในลักษณะที่ผันแปรตามความเข้มข้นที่เพิ่มขึ้น โดยไปลดระดับการแสดงออกของยีนไนตริกออกไซด์ซินเตสซึ่งเป็นเอนไซม์ที่สำคัญต่อการผลิตไนตริกออกไซด์ในไมโครเกลีย ดังนั้นผลการทดลองครั้งนี้ชี้ให้เห็นว่า การสะสมของธาตุเหล็กในไมโครเกลียภายใต้สภาวะที่มีการเสื่อมของเซลล์ประสาทอาจเป็นกลไกในการปกป้องเซลล์ประสาทต่อความเป็นพิษของไนตริกออกไซด์