# การศึกษาผลของยาดีเฟอร์ร็อกซามีนต่อการมีชีวิตรอดของเซลล์ในระบบประสาท และการตอบสนองของระบบภูมิคุ้มกันในเซลล์ไมโครเกลีย BV-2

**สุภาณี ชวนเชย[1,2] นวลจันทร์ จุฑาภักดีกุล[1]**
[1]ศูนย์วิจัยประสาทวิทยาศาสตร์ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล
[2]คณะกายภาพบำบัด มหาวิทยาลัยหัวเฉียวเฉลิมพระเกียรติ

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**บทคัดย่อ**

ไมโครเกลียเป็นเซลล์ในระบบภูมิคุ้มกันของระบบประสาทซึ่งเมื่อถูกกระตุ้นจะหลั่งสารก่อการอักเสบ ทำให้มีการอักเสบเรื้อรังซึ่งเป็นสาเหตุหนึ่งของโรคทางระบบประสาท ปริมาณเหล็กสะสมในร่างกายมากเกินไป เป็นปัจจัยหนึ่งที่ทำให้เกิดการอักเสบของเซลล์ในระบบประสาท ดีเฟอร์ร็อกซามีน (Deferoxamine; DFO) เป็นยาขับเหล็กที่ใช้กันแพร่หลายเพื่อกำจัดเหล็กส่วนเกินออกจากร่างกาย ช่วยลดอาการแทรกซ้อนจากภาวะเหล็กเกิน และลดการตายของเซลล์ประสาท อย่างไรก็ตาม การใช้ยา DFO ในปริมาณสูงต่อเนื่องเป็นเวลานานอาจทำให้เกิดภาวะพร่องออกซิเจนจนทำให้เซลล์ประสาทบาดเจ็บและตายได้ ข้อมูลเกี่ยวกับผลของยา DFO ต่อการมีชีวิตรอดของเซลล์ประสาทและเซลล์ไมโครเกลียยังไม่เคยมีรายงานมาก่อน งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของยา DFO ต่อการมีชีวิตรอดของเซลล์ในระบบประสาท โดยเพาะเลี้ยงเซลล์สายพันธุ์นิวโรบลาสโตมา (SH-SY5Y) และสายพันธุ์ไมโครเกลีย (BV-2) ในจานเพาะเลี้ยงที่เติม DFO ความเข้มข้น 25, 50 และ 100 µM เป็นเวลา 24 และ 48 ชั่วโมง จากนั้นตรวจสอบความอยู่รอดของเซลล์ทั้งสองชนิดรวมทั้งวัดระดับไซโตไคน์ IL-10 ซึ่งทำหน้าที่ต้านการอักเสบ ผลการทดลองพบว่าหลังจากได้รับ DFO นาน 24 ชั่วโมง เซลล์ทั้งสองชนิดมีชีวิตรอดน้อยลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม แต่เมื่อเวลาผ่านไป 48 ชั่วโมง พบว่าเซลล์ SH-SY5Y ยังคงมีอัตรารอดชีวิตน้อย ในขณะที่เซลล์ BV-2 มีแนวโน้มรอดชีวิตเพิ่มขึ้นและมีปริมาณไซโตไคน์ IL-10 เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม ผลการวิจัยนี้แสดงว่า DFO แม้ใช้ในปริมาณน้อยก็อาจทำให้เกิดการตายของเซลล์ประสาทและไมโครเกลียได้โดยเฉพาะใน 24 ชั่วโมงแรกหลังจากได้รับยา ดังนั้น การใช้ยา DFO ในผู้ป่วยที่จำเป็นต้องรับยานี้ควรใช้ด้วยความระมัดระวัง เพื่อหลีกเลี่ยงการทำลายเซลล์ในระบบประสาท นอกจากนั้นการที่เซลล์ไมโครเกลียรอดชีวิตเพิ่มขึ้นและหลั่งสารต้านการอักเสบหลังจากได้รับ DFO นาน 48 ชั่วโมง แสดงว่าไมโครเกลียอาจมีบทบาทช่วยต้านการอักเสบของเซลล์ประสาทในภาวะที่ขาดออกซิเจนและอาจเป็นเป้าหมายใหม่ในการรักษาโรคที่เกี่ยวกับการอักเสบเรื้อรังในระบบประสาท

**คำสำคัญ:** ดีเฟอร์ร็อกซามีน การมีชีวิตรอดของเซลล์ เซลล์นิวโรบลาสโตมา SH-SY5Y เซลล์ไมโครเกลีย BV-2 อินเตอร์ลิวคิน-10

**ผู้นิพนธ์ประสานงาน:**
**นวลจันทร์ จุฑาภักดีกุล**
ศูนย์วิจัยประสาทวิทยาศาสตร์ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล
25/25 ถนนพุทธมณฑล สาย 4 ตำบลศาลายา อำเภอพุทธมณฑล จังหวัดนครปฐม 73170
อีเมล: grnjgnew@gmail.com

# Effects of deferoxamine on the survival of the neuroblastoma SH-SY5Y cells and neuroimmune response in the BV-2 microglial cells

**Supanee Chounchay[1,2], Nuanchan Chutabhakdikul[1]**
[1]Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University
[2]Faculty of Physical Therapy, Huachiew Chalermprakiet University

## Abstract

Microglial are the resident immune cells in the central nervous system (CNS). They release cytokines and chemokines associate with inflammation and consequently lead to the neurological diseases. Iron overload in the CNS is one factor that triggers neuroinflammation. Deferoxamine (DFO) is an iron chelator widely used for removing excessive iron to protect neurons from iron overload. However, a high dose of DFO can induce oxygen depletion and hypoxic damage of neurons. It is still not clear how DFO has an impact on survival and death of cell in the nervous system. Therefore, the objective of this study is to investigate the effects of DFO on cell viability of neuron and microglia cells. The neuroblastoma (SH-SY5Y) and Microglia (BV-2) cell lines were cultured in a completed medium containing DFO at 25, 50, and 100 μM for 24 to 48 hours. Then, the cell viability and the expression of anti-inflammatory cytokine IL-10 were measured. The results showed that after 24 hours of DFO treatment, the cell viability of both cells were significantly decreased as compared to the control. Although the SH-SY5Y cell viability still decreases after 48 hours of DFO treatment, there is a trend to increases of BV-2 cell viability together with a significant increase in the level of IL-10 expression. The finding suggested that DFO treatment, even at low dosage, can induce neuronal and microglial cell death. Furthermore, enhance microglial cell survival and IL-10 expression indicated that the microglia might play an anti-inflammatory role following the hypoxic injury. Our results suggest that DFO should be carefully prescribed to avoid the adverse effects of DFO on hypoxia-induced cell death, especially within 24 hours after drug treatment. Finally, microglia might be a novel therapeutic target for the treatment of neurological diseases related to chronic neuroinflammation.

**Keywords:** deferoxamine, cell viability, SH-SY5Y, BV-2 cell, IL-10

**Corresponding Author:**
**Nuanchan Chutabhakdikul**
Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University
25/25 Phuttamonthon Sai 4 Road, Salaya Sub-district, Phuttamonthon District,
Nakhon Pathom Province 73170, Thailand.
E-mail: grnjgnew@gmail.com

## Introduction

Neuroinflammation and iron accumulation in the central nervous system (CNS) contributed to the pathology of neurological diseases[1,2]. Neuroinflammation induces neurotoxicity and free radical generation and consequently, lead to neuronal damage and death[3,4]. Iron is an essential micronutrition that plays an important role in oxygen transport, myelin synthesis, neurotransmitter synthesis, and neuronal metabolism[5-7]. However, when an iron overload occurs in the brain i.e., following the intracerebral hemorrhage, it can cause oxidative stress and cellular damage[8]. Previous studies reported that iron overload could contribute to the pathology of several neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's diseases[6,9].

Deferoxamine (DFO) is an iron chelator widely used in the clinic for removing excessive iron accumulation[10,11]. The DFO can promote iron homeostasis and reduce neuronal cell death by inhibit neuroinflammation[2]. Several lines of evidence consistently reported for the neuroprotective effects of DFO[10,11]. For example, clinical studies reported that intravenous infusion of DFO within 24 hours of onset (32 mg/kg/day, for 3 consecutive days) could reduce hematoma and edema volume in intracerebral hemorrhage patients[12]. However, DFO has adverse effects that contributed to cytotoxicity by inhibited DNA synthesis[13]. Besides, DFO induces hypoxia-inducible factor 1 alpha (HIF-1a) accumulation[14,15] and cause cell death[16]. The DFO stabilize HIF-1 alpha via prolyl hydroxylases (PHDs) pathway by depleting ferric iron (Fe2+)[15,17]. Recent studies reported that expression of HIF-1$\alpha$, a marker of hypoxia cells, in neural progenitor cells treated with 100 μM DFO for 24 hours[18]. Consequently, DFO has been used as the hypoxia mimetic agent to induced hypoxic-ischemic injury[14,18-19].

Microglia are the resident immune cells in the CNS. They release several cytokines and chemokines in response to neuroinflammatory signals. Neuroinflammation trigger morphological and functional changes of microglia from the resting state to the activated microglia. There are two phenotypes of activated microglial; 1) the proinflammatory microglia that release pro-inflammatory cytokines such as tumor necrosis factor (TNF-$\alpha$), interleukin (IL)-6, and IL-1$\beta$ and 2) the neuroprotective phenotypes that release anti-inflammatory cytokine such as IL-10 to promote neuronal cell restoration[20-22]. DFO plays a dual role in both neuroprotective and neuroinflammatory effects. Besides, several studies demonstrated DFO affects neuronal cell survival, however, the effects of DFO on microglial cell viability and function are still unclear.

## Objectives

This study aims to investigate the effects of DFO on cell viability of the neuroblastoma SH-SY5Y cells and the BV-2 microglial cells and the expression level of the anti-inflammatory cytokine (IL-10) in the BV-2 microglial cells.

## Materials and Methods

### Cell lines and reagents

The human SH-SY5Y neuroblastoma cell line was kindly provided by Dr. Wipawan Thangnipon. The immortalized murine microglia BV-2 cell was a kind gift from Dr. Stephen C. Noctor, MIND Institute, University of California, Davis, Sacramento, CA, USA. Deferoxamine mesylate (DFO; D9533) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT; M2128) were purchased from Sigma-Aldrich, Inc., USA. A mouse monoclonal anti - IL-10 (sc-365858) was purchased from Santa Cruz Biotechnology, Inc., USA.

### Cell lines culture

The SH-SY5Y and BV-2 cell were cultured in a complete medium consisted of Dulbecco's modified Eagle medium (DMEM) supplement with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C under a humidified atmosphere of 5% $CO_2$. At 80-90% confluence in T75 flasks, cells were harvested using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and plated onto a 96-well plate at a density of $1\times10^5$ cells/well.

### Deferoxamine Mesylate treatment

A stock DFO solution (50 mg/ml in distilled water) was prepared immediately prior to use. The culture medium was changed to a complete medium mixed with DFO at serial concentrations of 0, 25, 50, and 100 µM for 24 and 48 hours time incubation at 37°C under a humidified atmosphere of 5% $CO_2$. All experiments were used for cell viability assay, which determined the number of viable cells after DFO treatment.

### Cell viability assay

Cell viability was measured by a vital dye MMT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) for quantifying viable cells. MTT solution was prepared as a 5 mg/ml stock solution in phosphate-buffered saline (PBS) and kept in the dark. Cells were incubated with 0.5 mg/ml MTT at 37°C for 4 hours, and then the supernatant was removed and added 100 µl dimethyl sulfoxide (DMSO) to dissolve the blue formazan crystals. Cell viability was presented as the percentage of A570 nm of each sample relative to control measured by a microplate reader (SpectraMax®).

### Western blot analysis

Western blotting was used for detecting IL-10 expression in BV-2 cells. Proteins were extracted from BV-2 cell cultures in 100 µM DFO for 24 and 48 hours. A lysis buffer containing a phosphatase inhibitor was used for extraction and homogenization. Then, the supernatant was isolated from the lysate, and the protein concentration was measured using a Bradford assay. Thirty micrograms of proteins were resolved by electrophoresis on 10% acrylamide gels and electrotransferred onto a PVDF membrane. The membrane was incubated in blocking buffer for 1 hour, incubated with mouse monoclonal anti - IL-10 (sc-365858, 1:1000) overnight, rinsed 3 times in Tris-buffered saline and 0.1% Tween 20 (TBST) and then incubated with goat anti-mouse secondary antibody (sc-2005, 1:20,000)

for 1 hour. The immunoreactive signals were detected using a chemiluminescent reagent (ECL; Amersham Biosciences, Piscataway, NJ, USA), and images were scanned using an Azure Biosystems imaging system. The quantitative data by normalization to β-actin as a loading control were determined by ImageJ (National Institutes of Health, USA).

**Statistical analysis**

All data were presented as mean ± SEM of three independent experiments in triplicate. Statistical significance analysis was examined by one-way analysis of variance (ANOVA) followed by Bonferroni comparison tests using GraphPad Prism software 7.0, USA. *p*-values less than 0.05 were considered statistically significant.

## Results

**DFO treatment for 24 hours decreases cell viability of the SH-SY5Y cells and the BV-2 microglia cells**

SH-SY5Y and BV-2 cells were exposed to DFO for 24 hours. Figure 1A showed a significant decrease in SH-SY5Y cell viability at 25, 50, and 100 μM DFO concentration as compare to control (79.89±5.15%, 79.92±5.84%, and 79.70±4.39%, respectively). The BV-2 cell viability showed in Figure 1B, the results demonstrated a significant decrease in BV-2 cell viability at 25, 50, and 100 μM DFO concentration as compare to control (77.02±0.95%, 70.86±2.86%, and 68.26±5.46%, respectively).

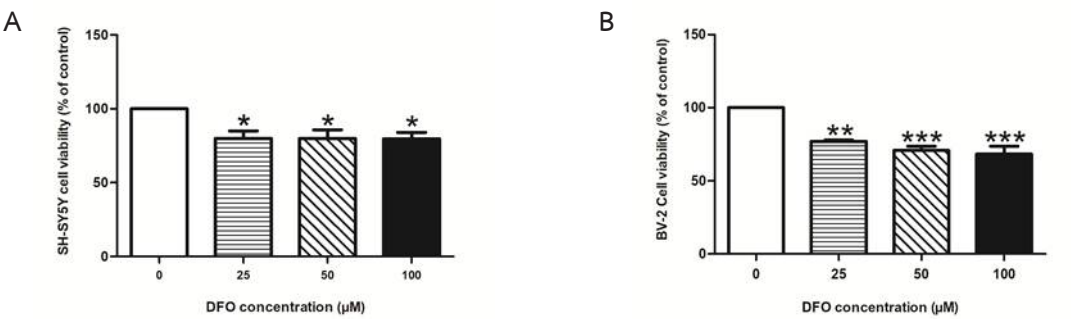


**Figure 1** Effect of twenty-four hours DFO exposure on cell viability. SH-SY5Y *(A)* and BV-2 *(B)* cells were treated with DFO at the final concentration 25, 50, and 100 μM for 24 hours. The results were obtained from three independent experiments represent the mean ± SEM, n=3 per group. **p*<0.05, ***p*<0.01, ****p*<0.001 as compared with the control group.

**DFO treatment for 48 hours decreases cell viability of the SH-SY5Y cells**

SH-SY5Y and BV-2 cells were exposed to DFO for 48 hours. The results of SH-SY5Y cell viability showed in Figure 2A, SH-SY5Y cell viability was a significant decrease at 25, 50, and 100 μM DFO concentration as compare to control (59.02±2.54%, 68.69±7.45%, and 61.66±10.04%, respectively). Figure 2B showed no significant differences in BV-2

cells at all concentrations as compare to control (114.1±16.96%, 118.3±24.08%, and 99.7±20.52%, respectively). However, the increasing trend of BV-2 cell viability is found.

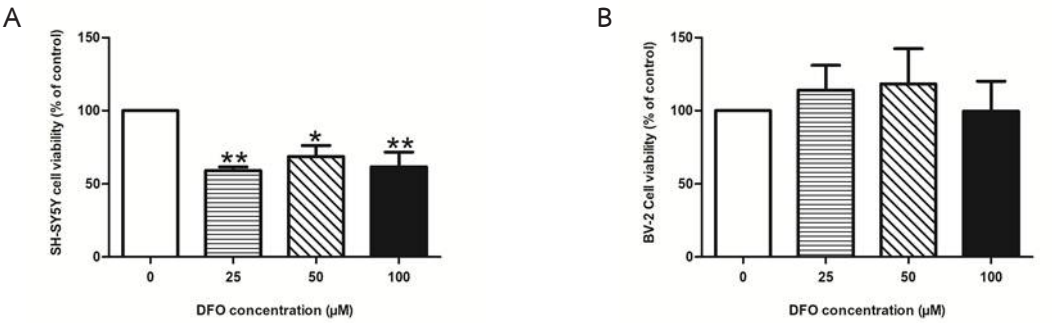


**Figure 2** Effect of forty-eight hours DFO exposure on cell viability. SH-SY5Y *(A)* and BV-2 *(B)* cells were treated with DFO at the final concentration 25, 50, and 100 μM for 48 hours. The results were obtained from three independent experiments represent the mean ± SEM, n=3 per group. **p*<0.05, ***p*<0.01 as compared with the control group.

**Comparison of DFO treatment time in SH-SY5Y and BV-2 cell viability**

When compare the DFO treatment time between the 24 and 48 hours in SH-SY5Y and BV-2 cells (Figure 3A and 3B), there were no significant differences in all concentration both SH-SY5Y and BV-2 cells, except at final concentration 25 μM DFO in SH-SY5Y cell. The results showed the SH-SY5Y cell viability was a significant decrease in 48 hours DFO treatment time as compared to 24 hours DFO treatment time.

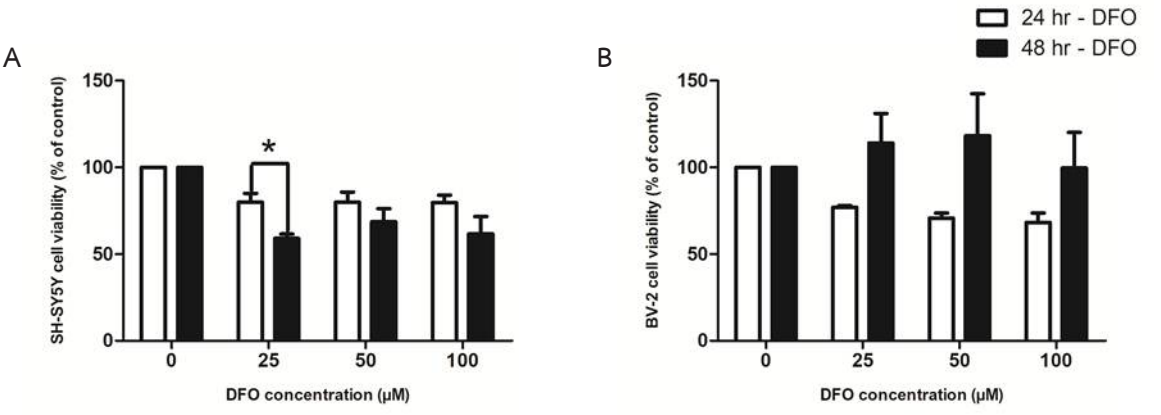


**Figure 3** Effect DFO exposure time on cell viability. SH-SY5Y *(A)* and BV-2 *(B)* cells were treated with DFO at the final concentration 0, 25, 50, and 100 μM for 24 and 48 hours. The results were obtained from three independent experiments represent the mean ± SEM, n=3 per group. **p*<0.05 show significant differences between 24 hr - DFO and 48 hr - DFO.

**DFO treatment for 48 hours increase IL-10 expression in BV-2 microglia cells**

The results showed the IL-10 expression in BV-2 cell after 100 μM DFO treatment for 24 and 48 hours. The level of IL-10 protein was measured by western blotting demonstrated a significant increase in BV-2 cell survival after 48 hours of DFO exposure as compared with the control and 24 hours of DFO exposure (Figure 4).

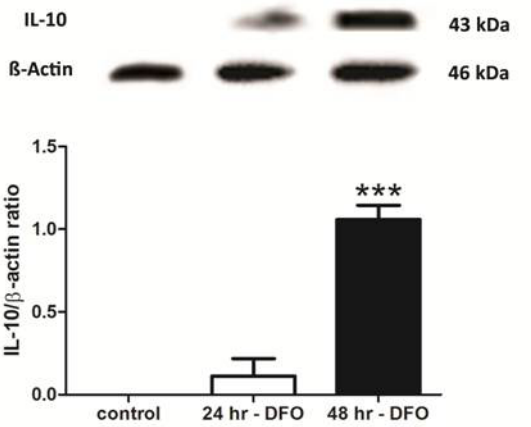


**Figure 4** Effect of DFO on IL-10 expression in BV-2 cell. BV-2 cells were exposed with 100 μM DFO for 24 (24 hr - DFO) and 48 hours (48 hr - DFO) compared to the control group. The upper panel displays the western blot analysis of IL-10 protein in the BV-2 cell. The lower panel displays the bar graph of quantitative results from two independent experiments in duplicate. All data represent the mean ± SEM of band densities/b-actin ratio, n=3 per group. ***$p<0.0001$ compared with the control and twenty-four group.

**Discussion**

DFO is an iron chelator for removing excessive iron in several neurological diseases that involve the neuroinflammation process. On the other hand, iron deficiency induces neurological disease and neuronal function impairment. Thus, the current study determined the role of DFO on neuronal and microglial cells. We have demonstrated that the SH-SY5Y cell viability was significantly decreased when exposed to 25 μM DFO for 24 and 48 hours. While BV-2 cell viability was significantly decreased when exposed to 24 hours and was not significantly different in the cell viability after DFO exposure for 48 hours, but the increasing trend was found.

DFO inhibited the autophagy - lysosomal pathway protected neuroblastoma cell apoptosis[23]. Rakshit and colleagues examined the effect of DFO treatment in SH-SY5Y induced neurotoxic cell by 6-Hydroxydopamine (6-OHDA). The SH-SY5Y cell viability was measured by MTT assay. They found a neuroprotective effect of 50 μM

to 500 μM DFO concentrations for 24 hours by increasing SH-SY5Y cell viability. However, they suggested that 100 μM and 250 μM DFO treatment showed a significantly increased cell viability, whereas at the highest concentration with 500 μM of DFO showed dramatically decreased cell viability[23]. Consequently, DFO plays a role in the neuroprotective effect. While the adverse effect of DFO can occur when the appropriated dose was used. The present study demonstrated reduced SH-SY5Y cell viability with increasing concentration of DFO as well as exposure time in healthy cell. Previous studies reported that the concentration of DFO in a dose-dependent manner upregulated a HIF-1a via cyclooxygenase-2 (COX-2) signaling pathway indicated inflammation[24]. Also, an iron level in the brain was reduced by iron chelator, 100 μM DFO, decreased brain-derived neurotrophic factor (BDNF) level contributed to the neuronal cell vulnerability to ischemic injury[25]. On the other hand, iron deficiency affects any brain area and neuronal function. A recent study found a ferritin level and lipid peroxidation were increased in the hippocampus indicated iron deficit and oxidative stress. Besides, dopamine metabolites including 3,4-dihydroxyphenylaceticacid (DOPAC) and homovanillic acid (HVA) in the striatum were decreased, whereas a monoamine oxidase and the HVA in the prefrontal cortex was increased[7]. Furthermore, iron deficit alters neuronal function contributed to cognitive impairments involved memory and learning because of the alteration of synaptic plasticity in several areas of the brain[26,27]. The iron chelator, DFO, inhibited calcium releasing to the postsynaptic membrane and N-methyl-D-aspartate (NMDA receptor) induced extracellular-signal-regulated kinase (ERK pathway) in primary hippocampal neurons, indicated diminished basal synaptic transmission related to learning impairment via long-term potentiation (LTP) in hippocampal CA1 neurons[27]. Therefore, an optimal dose of DFO can rescue the nervous system after injury. However, DFO treatment in normal condition induced a negative impact on the nervous system.

A previous study in the C57BL/6 mice showed that when microhemorrhages were induced at the posterior parietal cortex and treatment with DFO 100 mg/kg within 6 hours after the onset and every 12 hours for 3 days can help remove iron overload in the intracranial hemorrhage model. DFO administration reduced brain damage by diminished dendritic degeneration, blood-brain barrier disruption, and inhibited activated microglia and pro-inflammatory mediator[28]. A recent study suggested that the DFO regulated neuroinflammation and iron homeostasis after brain injury via p38 MAPK signaling, that contributed to reducing the release of reactive oxygen species and pro-inflammatory cytokines such as TNF-$\alpha$, IL-6 and IL-1$\beta$ on the third day after injury[2]. The effect of DFO on microglia cell deaths can be seen at 24-hour exposure. While, DFO could not alter

microglial cell viability at 48-hours exposure. Interestingly, IL-10 level in BV-2 cell exposed to 100 μM concentration of DFO for 48 hours was significantly increased indicate an anti-inflammatory cytokine releasing after hypoxia injury[22]. The DFO is a hypoxia mimetic agent that can induce HIF-1$\alpha$ in response to cell hypoxia[14,24,29] cause microglial cell death[16,30]. Previous study demonstrated the autophagic cell death of primary microglial cells after hypoxia 2, 4, 16, 24 and 48 hours, incubated in the hypoxia chamber (2%$O_2$/5% $CO_2$/93% $N_2$). Hypoxia suppressed microglial cell survival and promoted pro-inflammtory cytokines such as TNF-$\alpha$ and IL-8, especially the highest HIF-1$\alpha$ level was evaluated at hypoxia induction for 24 hours[16]. On the other hand, microglial cells enhanced neurogenesis at subventricular zone after hypoxia injury[31]. Moreover, hypoxia injury can induce IL-10 level increasing with time from 6, 24, 48, and 72 hours. The highest level of IL-10 was found at 48 hours after hypoxia and returned to the normal level after 1 week after hypoxia[32]. Thus, hypoxia is a biological signal that plays a dual role in the neurotoxicity effect and the neuroprotective effect involved neuroinflammation in response to microglial cells, depending on hypoxia environment such as severity and duration after brain damage[33-35].

## Conclusion

The results indicate that DFO, even at low doses, can induce cell death in the neuronal cells. Although neuronal cell death still occurred at 48 hours of DFO treatment, it is interesting that the survival rate of microglia trend to increased with an increased in the levels of IL-10. Our results indicate that microglia are more resilient than the neurons when DFO exposure. In addition, microglia might play an important role in supporting neuronal survival following iron deficiency.

## Recommendation

Our finding suggests that DFO could promote microglial cell viability under the hypoxic-ischemic condition and induce microglia releases of anti-inflammatory cytokine under hypoxia. Further experiments should be done to examine the survival rate of neuron and microglia at a longer period of DFO treatment using the neuronal and microglia co-culture model to examine the protective effect of microglia on neuronal survival under the hypoxic condition. We suggest that microglia might be a novel target for the treatment of neurological diseases associated with neuroinflammation.

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