

# Modulation of secretory factors by lipofundin contributes to its anti-neuroinflammatory effects

MING-SHAN CHEN<sup>1,2</sup>, CHIA-LIN HU<sup>1</sup>, SHIN-KUANG JIANG<sup>3</sup>, ZHI-YONG CHONG<sup>4</sup> and JUI-CHIEH CHEN<sup>4</sup>

<sup>1</sup>Department of Anesthesiology, Ditmanson Medical Foundation Chia-Yi Christian Hospital, Chiayi 60002;

<sup>2</sup>Department of Medical Laboratory Science and Biotechnology, Asia University, Taichung 41354;

<sup>3</sup>Department of Neurology, China Medical University Hospital, Taichung 404332; <sup>4</sup>Department of Biochemical Science and Technology, National Chiayi University, Chiayi 600355, Taiwan, R.O.C.

Received October 2, 2023; Accepted January 16, 2024

DOI: 10.3892/etm.2024.12456

**Abstract.** As the global population ages, the prevalence of neuroinflammatory diseases such as Alzheimer's disease, Parkinson's disease and stroke continues to increase. Therefore, it is necessary to develop preventive and therapeutic methods against neuroinflammatory diseases. Lipofundin is a lipid emulsion commonly used in clinical anesthetic solvents and nutritional supplements. Lipid emulsions have been shown to possess anti-inflammatory properties. However, the potential beneficial effect of lipofundin against neuroinflammation requires elucidation. In the present study, two cell models were used to investigate the efficacy of lipofundin against neuroinflammation. In the first model, BV2 mouse microglial cells were treated with lipopolysaccharide (LPS) to induce nitric oxide (NO) production as a model of neuroinflammation. In the second model, HMC3 human microglial cells were activated by LPS, and changes in the secretion of factors associated with inflammation were analyzed using Luminex xMAP<sup>®</sup> technology. Griess assay results revealed that lipofundin significantly prevented and treated LPS-induced NO production. An anti-neuroinflammatory effect was also observed in HMC3 cells, where lipofundin exhibited excellent preventive and therapeutic properties by reducing the LPS-induced expression and secretion of interleukin-1 $\beta$ . Notably, lipofundin also promoted the secretion of certain growth factors, suggesting a potential neuroprotective effect. These results demonstrate that, in addition to its role as a solvent for drugs and nutritional support, lipofundin may also have beneficial effects in alleviating the progression of neuroinflammation. These findings may serve as an important reference for future translational medicine applications.

## Introduction

The central nervous system is mainly composed of neurons and glial cells; the latter includes microglia which are immune system-associated cells accounting for 5-10% of all brain cells (1). Microglia in the brain have scavenger functions and participate in the protective mechanisms of the brain; however, under uncontrolled conditions, they can produce various inflammatory factors, including nitric oxide (NO), cytokines and chemokines, and lead to neuronal damage and brain cell death (2). A number of neurological diseases, including Alzheimer's disease (AD), stroke and other brain disorders, are associated with the activation of microglia. The secretion of inflammatory factors and reactive oxygen species (ROS) by microglia upon activation exerts a marked impact on the progression of these diseases (3,4).

During normal brain aging, age-associated inflammatory markers are highly expressed in the majority of elderly individuals, and these markers are associated with the loss of mental, cognitive and other complex behaviors that are characteristic of AD and Parkinson's disease (PD) (5). As the population ages, numerous countries around the world are gradually acquiring an increasingly elderly demographic. In the future, a marked proportion of elderly individuals are likely to experience a notable decline in neurological function and become more susceptible to neurological disorders and injuries. Therefore, it is urgently necessary to develop therapeutic approaches for the development of anti-neuroinflammatory medications.

Lipofundin is a lipid emulsion used in parenteral nutrition and vehicles. In a recent study, it was found that when neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA), lipofundin inhibited intracellular hypochlorous acid production and ERK activation to reduce PMA-stimulated neutrophil extracellular traps (NETs). It was also observed that lipofundin reduced *E. coli*-induced NETs in neutrophils through a ROS-independent pathway (6). In another study, in which *Staphylococcus aureus*-infected mouse RAW264.7 macrophages were tested, it was observed that lipofundin reduced ROS production and phagocytosis via the inhibition of JNK activation, thereby increasing bacterial survival (7). In the same macrophage model, lipofundin was also observed to reduce interleukin (IL)-1 $\beta$  secretion, ROS production and

---

*Correspondence to:* Professor Jui-Chieh Chen, Department of Biochemical Science and Technology, National Chiayi University, 300 Syuefu Road, Chiayi 600355, Taiwan, R.O.C.  
E-mail: jcc@mail.ncyu.edu.tw

**Key words:** lipid emulsions, lipofundin, neuroinflammation, secretome

phagocytosis (8). These studies have shown that lipofundin has excellent anti-inflammatory properties. However, whether lipofundin also has anti-neuroinflammatory properties remains largely unknown.

Secreted substances are important in intercellular communication and cell signaling, and play a critical role in the regulation of immune responses such as inflammation and defense against microbial pathogens (9). Abnormalities in secreted substances are associated with a wide variety of inflammatory diseases (10). Luminex biomarker multi-analyte profiling (xMAP<sup>®</sup>) technology was developed in the late 1990s as a high-throughput bioassay platform for the rapid, cost-effective and simultaneous analysis of multiple soluble analytes in a single small-volume sample (11). This technology enables the determination of soluble analyte profiles in biological samples derived from cell culture, animals or patients. This technology was used to determine the effect of lipofundin on secreted substances, and the results may serve as a reference for future translational medicine.

## Materials and methods

### *Experimental design*

**Murine microglial cell experiment.** The toxicity of lipofundin to murine microglial cells and its impact on the inflammatory response were investigated, via the examination of murine microglial cells in inactivated and lipopolysaccharide (LPS)-induced activated states.

**Experimental strategies.** In the preventive approach, murine microglial cells were pre-treated with lipofundin, followed by induction with LPS to activate the cells. In the therapeutic approach, murine microglial cells were treated with LPS to activate them, and the cells were subsequently treated with lipofundin.

**Measurement methods.** MTT and lactate dehydrogenase (LDH) assays were utilized to analyze the toxicity of lipofundin to murine microglial cells, and the impact of lipofundin on the inflammatory response was evaluated by the measurement of nitric oxide (NO) secretion and NO synthase 2 (NOS2) expression, categorized into preventive and therapeutic anti-inflammatory aspects.

**Human microglial cell experiment.** The results obtained from the murine microglial cell experiments were validated using human microglial cells.

**Experimental strategies.** Considering that human microglial cells do not secrete NO upon activation, a suitable marker was identified by the analysis of secreted proteins using a Luminex multiplex cytokine assay. The results suggested IL-1 $\beta$  as a suitable molecule. Preventive and therapeutic approaches were used for the human microglial cells as described for the murine cells.

**Measurement methods.** MTT and LDH assays were employed to analyze the toxicity of lipofundin to human microglial cells, and reverse transcription-quantitative PCR (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA) were used to measure the expression of IL-1 $\beta$  and assess the impact of lipofundin on the inflammatory response. This impact was categorized into preventive and therapeutic anti-inflammatory aspects.

This comprehensive experimental strategy was designed to explore the effects of lipofundin on microglia in mice and human cells, addressing toxic and anti-inflammatory responses in a variety of settings.

**Cell culture and drug treatment.** BV2 mouse microglia cells were kindly provided by Dr Yuh-Chiang Shen from the National Research Institute of Chinese Medicine (Taipei, Taiwan). HMC3 human microglia cells were purchased from the American Type Culture Collection (CRL-3304). The two cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin at 37°C in a humidified chamber with 5% CO<sub>2</sub>. The treatment of BV2 and HMC3 cells with lipofundin was carried out using two different conditions: i) Preventive approach, in which BV2 cells underwent pretreatment with specified concentrations of lipofundin (0-1,000  $\mu$ g/ml) for 1 h followed by co-treatment with 250 ng/ml LPS for 24 h, and HMC3 cells were pretreated with 62.5  $\mu$ g/ml lipofundin for 1 h, followed by co-stimulation with 250 ng/ml LPS for 24 h; and ii) therapeutic approach, in which BV2 cells were pre-stimulated with 250 ng/ml LPS for 1 h, followed by co-treatment with increasing doses of lipofundin (0-1,000  $\mu$ g/ml) for 24 h, and HMC3 cells were pre-stimulated with 250 ng/ml of LPS for 1 h, followed by co-treatment with 62.5  $\mu$ g/ml lipofundin for 24 h at 37°C.

**MTT assay.** Cell viability was determined by MTT assay. Briefly, cells were treated with MTT reagent (5 mg/ml in PBS) and incubated for 4 h at 37°C. Then, the supernatant was aspirated and 100  $\mu$ l DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at a wavelength of 550 nm using a microplate reader, and the background value at a wavelength of 750 nm was subtracted. Cell viability was determined as the percentage relative to that of untreated cells.

**Griess assay.** NO production was determined by the measurement of its stable end-product, nitrite, using a Griess reagent. Briefly, 100  $\mu$ l cell supernatant was added to a 96-well plate and mixed with 100  $\mu$ l Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 5% phosphoric acid). The mixture was incubated at room temperature for 10 min to allow for color development. The absorbance at 540 nm was measured using a microplate reader and nitrite concentrations were determined with reference to a calibration curve prepared with sodium nitrite standards.

**LDH assay.** The potential cytotoxic effect of lipofundin on cells was assessed by quantifying the leakage of LDH into the extracellular fluid. The supernatant from each sample was collected for the analysis of LDH using a Cytotoxicity Detection Kit (cat. no. 11644793001, Sigma-Aldrich; Merck KGaA). The absorbance of each sample was measured at 490 nm using a microplate reader. LDH release was calculated as a percentage according to the following equation: LDH release (%)=(LDH activity in the medium / total LDH activity) x100, where total LDH activity is the sum of LDH in the medium and LDH in the cells.

**Western blot analysis.** Cell lysis was performed using RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate) supplemented with a protease inhibitor cocktail (cat. no. P8340), all purchased from Sigma-Aldrich (Merck KGaA). The protein concentration was determined using a Pierce BCA Protein Assay kit (cat. no. 23235; Thermo Fisher Scientific, Inc.). Equal amounts of total protein (35  $\mu$ g/lane) were separated by SDS-PAGE with an 8% gel. The gels were subsequently transferred onto PVDF membranes (MilliporeSigma). Following transfer, the membranes were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature for 1 h. Next, the membranes were incubated overnight at 4°C with NOS2 (1:1,000; cat. no. 22226-1-AP; Proteintech) and  $\alpha$ -tubulin (1:10,000; cat. no. 05-829; MilliporeSigma) primary antibodies according to the manufacturer's instructions. After washing with TBST, the membranes were incubated with appropriate HRP-conjugated secondary antibodies: Goat anti-rabbit IgG (cat. no. 20202; Leadgene Co., Ltd.) and goat anti-mouse IgG (cat. no. 115-035-003; Jackson ImmunoResearch Laboratories, Inc.), both diluted 1:5,000, at room temperature for 1 h. Finally, the membranes were washed with TBST and the bound antibodies were visualized using an Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma) and autoradiography.

**Collection of conditioned media.** To understand the secretion of cytokines, chemokines and growth factors, HMC3 cells were cultured in the presence of 250 ng/ml LPS for 24 h. Additionally, HMC3 cells were pretreated with 62.5  $\mu$ g/ml lipofundin for 1 h and then co-stimulated with 250 ng/ml LPS for 24 h. The supernatants were subsequently collected and concentrated for analysis using multiplex immunoassays. The conditioned media were concentrated to a volume of 150-200  $\mu$ l using Amicon Ultra-15 centrifugal filters with a 3-kDa cut-off (MilliporeSigma) at 4,000  $\times$  g and 4°C for ~12 h.

**Luminex multiplex cytokine assay.** To analyze the levels of cytokines, chemokines and growth factors in the HMC3 cell supernatants, Luminex-based Milliplex xMAP technology with a 48-plex Human Cytokine/Chemokine Magnetic Bead Panel (cat. no. HCYTA-60K; MilliporeSigma) was employed according to the manufacturer's instructions. The cells were divided into three groups, namely the untreated, LPS-treated and lipofundin + LPS-treated groups.

**RT-qPCR.** Total RNA was isolated from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Subsequently, cDNA synthesis was performed using M-MLV reverse transcriptase, Oligo and dNTP Mix (Takara Bio, Inc.) at 37°C for 30 min, followed by heating to 85°C for 5 sec. qPCR was then conducted with specific gene primers using the MyGo PCR Detection System (IT-IS Life Science, Ltd.), according to the manufacturer's guidelines. The qPCR reactions were performed using KAPA SYBR Fast qPCR Master Mix kit (cat. no. KR0389; KAPA Biosystems, Inc.), following the recommended protocol. The qPCR reaction included an initial denaturation step at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 2 min and annealing/extension at 60°C for 30 sec. The

comparative Cq ( $2^{-\Delta\Delta Cq}$ ) method was utilized to calculate relative gene expression, with the normalization of the target gene to GAPDH (12). The primer sequences used were as follows: IL-1 $\beta$ , forward: 5'-ATGATGGCTTATTACAGTGGCAA-3' and reverse: 5'-GTCGGAGATTTCGTAGCTGGA-3'; GAPDH, forward: 5'-CACCCATGGCAAATTCATGGCA-3'; and reverse: 5'-TCTAGACGGCAGGTCAGGTCCACC-3'.

**ELISA.** The concentrations of IL-1 $\beta$  in the cell supernatants were detected using an ELISA kit (cat. no. KTE6013; Abbkine Scientific Co., Ltd.) according to the manufacturer's protocol.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard error of the mean and were obtained from a minimum of three independent experiments. Statistical analysis was conducted using GraphPad Prism 6.0 software (Dotmatics). One-way analysis of variance with Tukey's post hoc test for multiple comparisons was used for the comparison of differences among groups.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

**Cytotoxicity and activating effects of lipofundin on BV2 cells in the absence of LPS stimulation.** Firstly, the cytotoxicity of lipofundin and its activating effects in BV2 cells were analyzed without LPS stimulation. BV2 cells were treated with lipofundin at different concentrations (0-1,000  $\mu$ g/ml) for 24 h, followed by analysis using an MTT assay. As shown in Fig. 1A, significant cell death was observed only at lipofundin concentrations  $>125$   $\mu$ g/ml. These results were validated using an LDH assay, as shown in Fig. 1B. Treatment with lipofundin at concentrations  $\leq 125$   $\mu$ g/ml did not induce cytotoxic effects in the BV2 cells, consistent with the results of the MTT assay. Additionally, the Griess assay was performed to investigate whether lipofundin has the potential to activate inflammation. As shown in Fig. 1C, lipofundin induced a significant inflammatory response in BV2 cells only at concentrations  $\geq 250$   $\mu$ g/ml.

**Preventive effects of lipofundin against LPS-induced inflammation.** Next, the ability of lipofundin pre-treatment to attenuate LPS-induced inflammatory responses in BV2 cells was analyzed. BV2 cells were pre-treated with lipofundin (0-1,000  $\mu$ g/ml) for 1 h and then co-stimulated with LPS (250 ng/ml) for 24 h. The assessment of cell viability using the MTT assay showed that high concentrations of lipofundin (500 and 1,000  $\mu$ g/ml) significantly decreased BV2 cell viability (Fig. 1D). Notably, during LPS activation, BV2 cells exhibited heightened tolerance to lipofundin toxicity, with significant cytotoxic effects observed only at concentrations  $\geq 500$   $\mu$ g/ml. These results were validated using the LDH assay, the results of which were consistent with the MTT findings. In addition, treatment with 62.5  $\mu$ g/ml lipofundin was observed to reduce LDH release, indicating that it inhibited cell death in LPS-activated BV2 cells (Fig. 1E). The Griess assay was also utilized to investigate whether lipofundin was able to prevent the inflammation induced by LPS in BV2 cells. As shown in Fig. 1F, treatment with lipofundin in the concentration range of 62.5-250  $\mu$ g/ml significantly reduced NO production compared with that in cells treated with LPS alone, with the

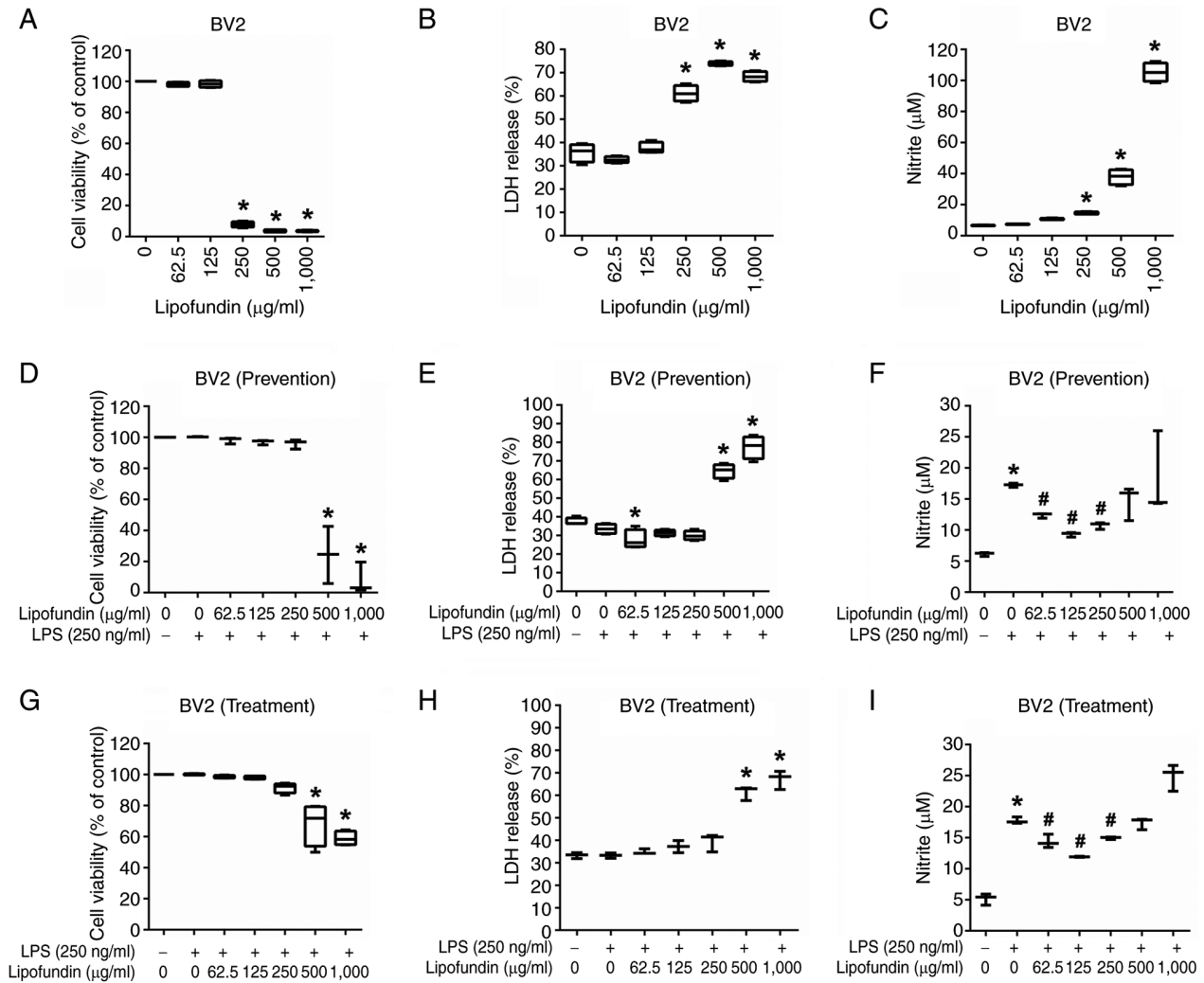


Figure 1. Effects of lipofundin on the viability and nitric oxide generation of BV2 cells with or without LPS. (A-C) BV2 cells were treated with increasing concentrations of lipofundin for 24 h. The cytotoxic effect of lipofundin on the cells was assessed using (A) MTT and (B) LDH assays. (C) Activation of the BV2 cells was determined using the Griess assay, via the measurement of nitrite production. (D-F) Preventive effect of lipofundin on neuroinflammation in BV2 cells. Cells were pretreated with the indicated concentrations of lipofundin for 1 h, followed by co-treatment with 250 ng/ml LPS for 24 h. Cytotoxicity was assessed using (D) MTT and (E) LDH assays. (F) Activation of the BV2 cells was also determined by the measurement of nitrite production using the Griess assay. (G-I) Therapeutic effect of lipofundin on neuroinflammation in BV2 cells. BV2 cells were pre-stimulated with 250 ng/ml LPS for 1 h, followed by co-treatment with increasing doses of lipofundin for 24 h. Subsequently, cell viability was evaluated using (G) MTT and (H) LDH assays, while (I) cell activation was determined using Griess analysis. \* $P < 0.05$  compared with the untreated control group; # $P < 0.05$  compared with the LPS-treated group. LPS, lipopolysaccharide; LDH, lactate dehydrogenase.

maximal anti-inflammatory effect observed at a concentration of 125  $\mu\text{g/ml}$ . During the activation of BV2 cells, NOS2 expression is highly upregulated, leading to the generation of a large amount of NO (13). To evaluate the activation status of BV2 cells, the expression of NOS2 was assessed using western blot analysis. As shown in Fig. 2A, pre-treatment with lipofundin for 1 h caused a dose-dependent reduction in NOS2 protein expression following LPS stimulation.

**Therapeutic effects of lipofundin on LPS-induced inflammation.** The therapeutic effects of lipofundin were also investigated, when used to treat BV2 cells following LPS activation. The results of MTT and LDH assays (Fig. 1G and H) revealed that LPS-pre-stimulated BV2 cells exhibited cytotoxicity when treated with lipofundin at concentrations of 500 and 1,000  $\mu\text{g/ml}$ . Due to prior LPS activation, the cells

exhibited increased tolerance to lipofundin toxicity. Even in LPS-primed BV2 cells, lipofundin effectively reduced inflammation, with a maximal anti-inflammatory effect at a concentration of 125  $\mu\text{g/ml}$  (Fig. 1I). To further confirm the therapeutic effects of lipofundin on LPS-induced activation, western blot analysis was performed to examine the expression of NOS2. The results revealed a dose-dependent reduction in NOS2 expression in LPS-induced cells with increasing concentrations of lipofundin treatment (Fig. 2B). These results suggest that lipofundin has the potential to prevent the occurrence of inflammation and exhibits therapeutic effects on existing inflammatory responses.

*Exploring the cytotoxicity of lipofundin and its influence on the secreted protein profile of HMC3 cells under LPS stimulation.* To better understand the effects of lipofundin



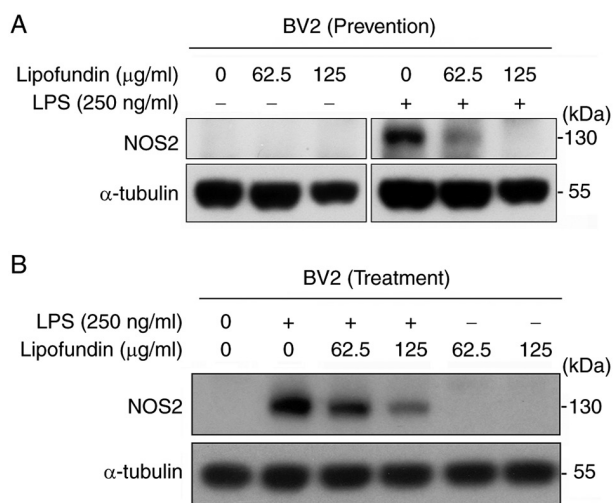


Figure 2. Protective and therapeutic effects of lipofundin as an anti-neuro-inflammatory agent assessed via measurement of the expression of NOS2 in LPS-induced BV2 cells. (A) BV2 cells were pretreated with lipofundin at concentrations of 0, 62.5 and 125 µg/ml for 1 h, followed by co-stimulation with or without 250 ng/ml LPS for 24 h. (B) BV2 cells were prestimulated with or without 250 ng/ml LPS for 1 h, and then co-treated with lipofundin (0, 62.5 or 125 µg/ml) for 24 h. The expression level of NOS2 was determined by western blot analysis using α-tubulin as a loading control. NOS2, nitric oxide synthase 2; LPS, lipopolysaccharide.

on human cells, the human microglial cell line HMC3 was analyzed. The cytotoxicity of lipofundin to HMC3 cells was evaluated using MTT and LDH assays. The results demonstrate that treatment with lipofundin at a concentration of 62.5 µg/ml had no significant impact on the viability of HMC3 cells (Fig. 3A and B). To understand the effects of LPS and lipofundin on the secretion of various factors associated with inflammation, including cytokines, chemokines and growth factors in HMC3 cells, a Luminex multiplex cytokine assay was conducted to analyze HMC3 cell culture supernatants. The results showed a >5-fold increase in the secretion levels of various factors under LPS treatment in HMC3 cells, including: granulocyte colony-stimulating factor (G-CSF; 11.02-fold), granulocyte-macrophage colony-stimulating factor (13.63-fold), growth related oncogene α (9.23-fold), IL-1β (16.60-fold), IL-17A (9.83-fold) and interferon-γ-inducible protein 10 (IP-10; 7.41-fold). However, pretreatment of the HMC3 cells with lipofundin effectively reduced LPS-induced secretion by >3-fold for factors including IL-1β and IL-17A, the secretion levels of which were 0.25- and 0.29-fold of those in cells treated with LPS alone. Also, when the HMC3 cells were pretreated with lipofundin, certain secretion levels were increased >3-fold compared with LPS stimulation alone, including G-CSF (3.39-fold), IL-12 (p70) (3.35-fold) and IP-10 (4.20-fold) as shown in Fig. 3C and Table I. These results suggest that lipofundin inhibits neuroinflammation in human microglia cells and promotes the secretion of substances such as growth factors that may have a neuroprotective effect.

*Lipofundin effectively suppresses the pro-inflammatory cytokine IL-1β when used for prevention or treatment.* The results from the Luminex multiplex cytokine assay demonstrated that the secretion of IL-1β by HMC3 cells was significantly increased upon LPS treatment. Therefore, the

expression and secretion of IL-1β were analyzed to evaluate the anti-inflammatory effect of lipofundin on HMC3 cells. As shown in Fig. 4A, RT-qPCR analysis revealed that lipofundin significantly downregulated the expression of IL-1β mRNA in both preventive and therapeutic cell models. These findings were further validated via ELISA, which demonstrated that lipofundin effectively and significantly reduced the secretion of IL-1β in both the preventive and therapeutic models (Fig. 4B).

## Discussion

In the present study, the selection of doses was carefully considered to ensure an appropriate range of lipofundin concentrations, as an excessive dose may induce cell toxicity while an insufficient dose may fail to elicit an effective response. Non-activated mouse BV2 cells were first treated with different concentrations of lipofundin to examine its impact on cell toxicity. The results indicated that 250 µg/ml lipofundin exhibited cytotoxic effects. This concentration also triggered the activation of BV2 cells, suggesting that, for inflammation prevention, the dose of lipofundin should be <250 µg/ml. In BV2 cells with LPS-induced activation, the cytotoxic concentration of lipofundin increased to 500 µg/ml. At this concentration, the effective reduction of LPS-induced BV2 cell activation was observed, indicating that for anti-inflammatory treatment the concentration of lipofundin should be <500 µg/ml. In non-activated human HMC3 cells, treatment with 62.5 µg/ml lipofundin showed no cytotoxicity. To ensure a safer dosage, 62.5 µg/ml lipofundin was selected for preventive and therapeutic inflammation experiments. The findings for these concentrations provide a foundation for cellular research and offer crucial references for translational medical applications.

Lipofundin is a widely used lipid emulsion for intravenous injection, primarily employed for drug solubilization and the provision of nutritional support through infusion. Generally, when used at the correct dosage and in appropriate contexts, lipofundin is considered relatively safe. However, the results of the cell experiments in the present study reveal a dual effect of lipofundin according to its concentration. At lower concentrations, therapeutic effects were observed, which manifested as significant anti-inflammatory properties. This suggests that within this lower dosage range, lipofundin may modulate or inhibit pathways associated with neuroinflammation, thereby demonstrating therapeutic potential. Conversely, at higher concentrations, lipofundin exhibited toxic effects. This toxicity may be associated with the triggering of alternative cellular responses or toxic pathways at elevated dosages, resulting in adverse effects. Additionally, it was observed that higher doses led to marked turbidity in the culture medium, which may have interfered with subsequent analyses. These observations highlight the dose-dependence of the effects of lipofundin, emphasizing that careful consideration is necessary when selecting an appropriate dose to ensure therapeutic efficacy without adverse effects. Further research focusing on elucidation of the molecular mechanisms of lipofundin at different concentrations is necessary, to gain a deeper understanding of its impact at the cellular and tissue levels, and ensure its safety in clinical applications.

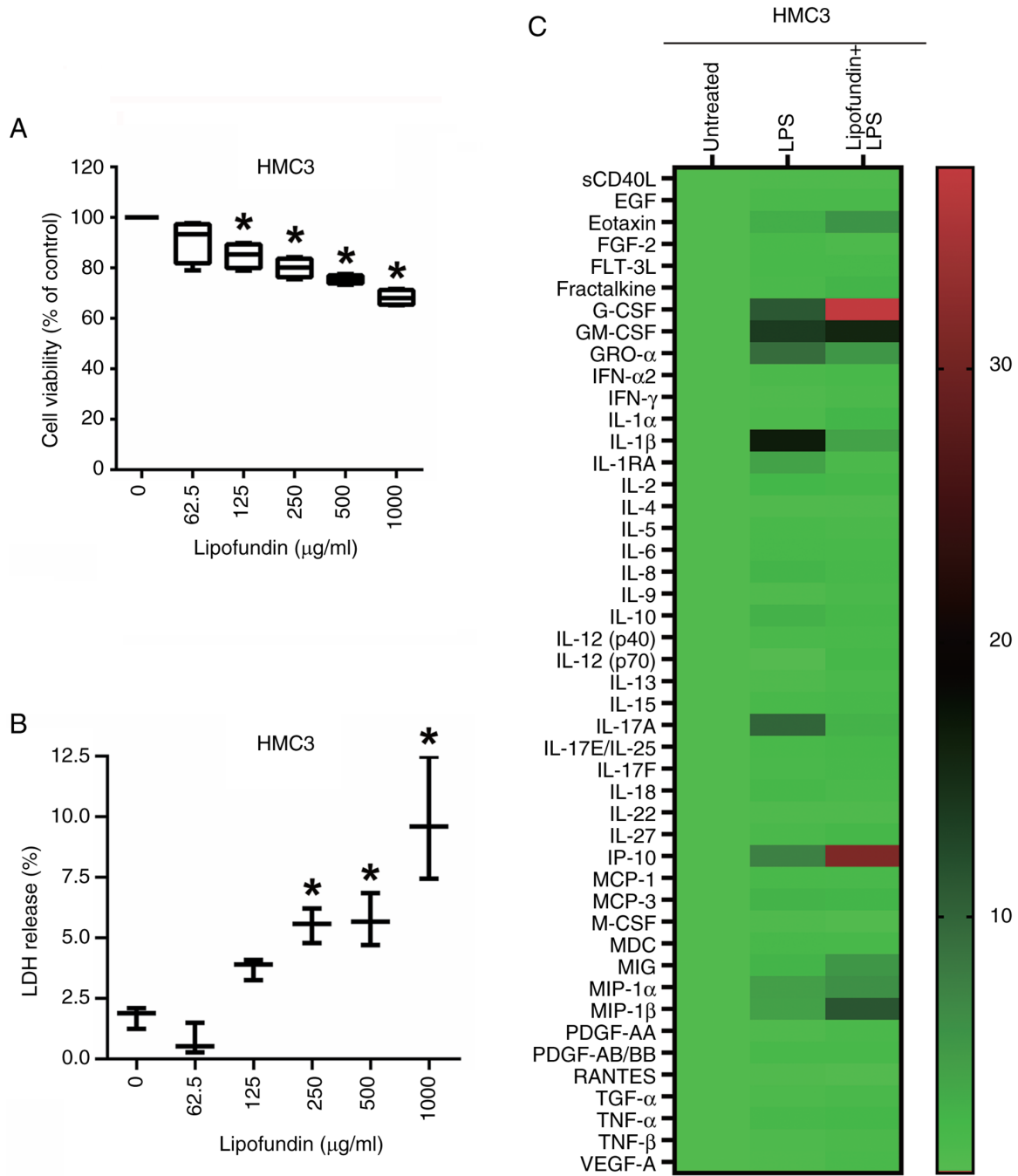


Figure 3. Cytotoxic effect of lipofundin on HMC3 cells and its secretion profile upon LPS stimulation. (A and B) Cells were treated with increasing concentrations of lipofundin for 24 h. The cytotoxicity of lipofundin was determined by (A) MTT and (B) LDH assays. \*P<0.05 compared with the untreated control group. (C) Heatmap depicting the impact of lipofundin on factors associated with inflammation in HMC3 cells pretreated with 62.5 µg/ml lipofundin for 1 h and then co-stimulated with 250 ng/ml LPS for 24 h. Conditioned media from these cells and untreated or LPS-stimulated control cells were analyzed using a Luminex multiplex assay. The heatmap displays the relative expression levels, with color intensity indicating the magnitude. LPS, lipopolysaccharide; LDH, lactate dehydrogenase; sCD40L, soluble CD40 ligand; EGF, epidermal growth factor; FGF, fibroblast growth factor; FLT-3L, Fms-related tyrosine kinase 3 ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GROα, growth related oncogene α; IFN, interferon; IL, interleukin; IP, IFN-γ-inducible protein; MCP, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MDC, macrophage-derived cytokine; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

The main clinical uses of lipid emulsions are in nutritional support, detoxification and as solvents for anesthetics. Clinical nutritional support may be administered enterally or parenterally. In patients whose gastrointestinal function is severely impaired, lipid emulsions can provide the necessary nutrition via parenteral administration to promote recovery and improve

the prognosis of the patient (14). Lipid emulsions can cross the blood-brain barrier due to their lipid solubility. A number of studies have reported that lipid emulsions have neuroprotective effects in mouse models of ischemic stroke (15-17). It has also been reported that ω-3 polyunsaturated fatty acids are able to regulate the functions of astrocyte and microglia,

Table I. Effect of lipofundin on the concentrations of cytokines, chemokines and growth factors in LPS-activated HMC3 cells as revealed by Luminex multiplex cytokine assay.

Analyte	Concentration, pg/ml		
	Untreated	LPS	Lipofundin + LPS
sCD40L	401.62	481.14	498.44
EGF	8.39	13.16	13.85
Eotaxin	2,059.03	6,504.67	11,509.91
FGF-2	555.94	992.80	782.12
FLT-3L	122.11	226.03	236.42
Fractalkine	617.33	1003.70	1626.70
G-CSF	11.90	131.12	444.31
GM-CSF	116.33	1,585.81	1,825.56
GRO $\alpha$	1037.37	9573.20	5519.04
IFN- $\alpha$ 2	28.72	43.30	52.39
IFN- $\gamma$	7.86	9.58	11.81
IL-1 $\alpha$	12.06	16.73	31.29
IL-1 $\beta$	10.59	175.85	44.22
IL-1RA	3.37	14.09	5.18
IL-2	2.80	7.00	6.12
IL-3	ND	ND	ND
IL-4	19.69	23.10	24.23
IL-5	1.09	1.96	1.62
IL-6	7,348.58	14,338.89	13,782.77
IL-7	ND	ND	ND
IL-8	3,914.12	10,558.17	7,993.79
IL-9	7.69	9.35	13.22
IL-10	3.56	10.38	7.54
IL-12 (p40)	31.07	48.22	56.14
IL-12 (p70)	10.69	6.93	23.22
IL-13	127.36	157.96	214.64
IL-15	366.98	652.46	740.22
IL-17A	7.75	76.20	22.14
IL-17E/IL-25	49.12	86.27	104.82
IL-17F	9.08	14.92	18.18
IL-18	1.44	3.10	2.32
IL-22	137.18	164.92	163.81
IL-27	1865.87	3035.15	4210.36
IP-10	71.22	527.81	2216.41
MCP-1	12031.75	21478.41	20896.11
MCP-3	45.72	124.21	119.16
M-CSF	2,132.59	2,591.80	2,055.54
MDC	2.42	4.65	4.58
MIG	36.21	97.73	194.07
MIP-1 $\alpha$	98.91	451.09	602.45
MIP-1 $\beta$	43.83	192.54	494.33
PDGF-AA	3,660.79	4,607.03	5,844.00
PDGF-AB/BB	763.71	1,435.92	1,435.05
RANTES	4,956.10	5,385.24	4,140.58
TGF- $\alpha$	100.24	141.58	166.93
TNF- $\alpha$	56.12	114.07	129.30
TNF- $\beta$	140.10	184.50	209.64

Table I. Continued.

Analyte	Concentration, pg/ml		
	Untreated	LPS	Lipofundin + LPS
VEGF-A	17,102.51	21,631.76	29,547.76

Human cytokines/chemokines/growth factors were quantified by Luminex multiplex cytokine assay. LPS, lipopolysaccharide; sCD40L, soluble CD40 ligand; EGF, epidermal growth factor; FGF, fibroblast growth factor; FLT-3L, Fms-related tyrosine kinase 3 ligand; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO $\alpha$ , growth related oncogene  $\alpha$ ; IFN, interferon; IL, interleukin; IP, IFN- $\gamma$ -inducible protein; MCP, monocyte chemoattractant protein; M-CSF, macrophage colony-stimulating factor; MDC, macrophage-derived cytokine; MIG, monokine induced by IFN- $\gamma$ ; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; ND, not determined.

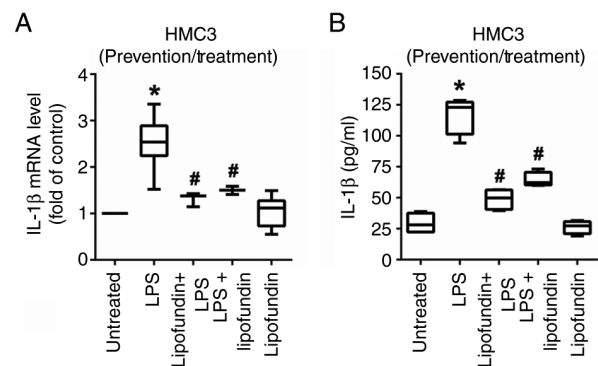


Figure 4. Protective and therapeutic effects of lipofundin on neuroinflammation in HMC3 cells evaluated by the expression and secretion of IL-1 $\beta$ . In the lipofundin + LPS group, cells were pre-treated with 62.5  $\mu$ g/ml lipofundin for 1 h, followed by co-stimulation with 250 ng/ml LPS for 24 h to evaluate the protective effect. In the LPS + lipofundin group, cells were pre-stimulated with 250 ng/ml LPS for 1 h, followed by treatment with 62.5  $\mu$ g/ml of lipofundin to evaluate the therapeutic effect. Untreated cells and cells treated only with LPS were included as negative and positive controls, respectively. (A) Expression of IL-1 $\beta$  mRNA was analyzed using reverse transcription-quantitative PCR, with GAPDH serving as the internal control. (B) Secretion levels of IL-1 $\beta$  in the cell culture supernatant were analyzed using an enzyme-linked immunosorbent assay. \* $P$ <0.05 compared with the untreated control group; # $P$ <0.05 compared with the LPS-treated group. LPS, lipopolysaccharide; IL, interleukin.

improve neuronal survival and attenuate ischemic stroke injury in a rat model of stroke using transient middle cerebral artery occlusion, suggesting that they have potential in neuroprotective and anti-inflammatory applications (18). In addition,  $\omega$ -3 polyunsaturated fatty acids were observed to attenuate microglia-induced inflammation through the high mobility group box 1/Toll-like receptor 4/NF- $\kappa$ B pathway in a traumatic brain injury-induced rat brain injury model (19). Furthermore, in a rat model of spinal cord injury, it was found that the intravenous injection of  $\omega$ -3 polyunsaturated fatty

acids immediately after injury regulated neuropathic inflammation and reduced neuronal damage (20).

A recent study investigated LPS-induced neuroinflammation and loss of learning and memory in C57BL/6J mice, and found that supplementing the diet of the mice with an astaxanthin-containing emulsion effectively ameliorated the cognitive, learning and memory impairment caused by inflammation, suggesting the potential use of the emulsion as a food or medicine for the treatment of clinical neuroinflammation (21). Another study used cecal ligation and perforation to induce sepsis in rats, which led to neurocognitive impairment. It was found that the administration of a fish oil-enriched lipid emulsion to the rats modulated neuroinflammation and effectively prevented long-term cognitive impairment after sepsis (22). In addition, previous studies have shown that  $\omega$ -3 polyunsaturated fatty acids have a beneficial effect on PD via the inhibition of proinflammatory cytokine release, promotion of neurotrophic factor expression, recovery of mitochondrial function and membrane fluidity, reduction of oxidant production levels, maintenance of  $\alpha$ -synuclein proteostasis, calcium homeostasis and axonal transport, and reduction of endoplasmic reticulum stress (23).

The present study revealed that lipofundin increases the secretion of G-CSF, and previous studies have indicated that G-CSF has a role in the promotion of M2 macrophage polarization (24,25). This suggests that the elevated secretion of G-CSF may have anti-inflammatory effects. A previous study has shown that intravenous lipid emulsion and G-CSF each have neuroprotective effects and can effectively improve memory and learning in an ischemic rat model, with a combination of the lipid emulsion and G-CSF being more effective than either alone (26). G-CSF is a glycoprotein produced by macrophages and endothelial cells, which stimulates the bone marrow to produce granulocytes and stem cells. A number of previous studies suggest that G-CSF reduces infarct size and improves brain function after ischemia (27-29).

The present study has certain limitations. Firstly, *in vitro* models comprising BV2 and HMC3 cell lines were utilized, which may not comprehensively reflect the complex processes of neuroinflammation *in vivo*. Therefore, further validation in animal models and clinical trials is necessary for translating the findings of the present study into practical therapeutic applications. Secondly, using the data derived from the Luminex multiplex cytokine assay, a more thorough exploration and validation is required to elucidate how lipofundin regulates secreted factors and its involvement in associated functions. Finally, despite this initial exploration into the impact of lipofundin on the modulation of neuroinflammation, further in-depth research is imperative to elucidate the precise molecular mechanisms and evaluate the potential therapeutic effects of lipofundin in neuroinflammatory diseases.

In conclusion, the present study investigated the preventive and therapeutic potential of lipofundin in inflammation. The results demonstrate that lipofundin not only prevented LPS-induced inflammatory responses in microglial cells but also exhibited therapeutic effects in cells that were already activated. These research findings highlight the potential efficacy of lipofundin as a modulator of neuroinflammatory responses, particularly its significant inhibitory effect on

IL-1 $\beta$ . This provides a beneficial theoretical foundation for the treatment of neurological disorders.

### Acknowledgements

Not applicable.

### Funding

The present study was supported by Ditmanson Medical Foundation Chia-Yi Christian Hospital (grant no. R111-69).

### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

MSC and JCC conceived and designed the study. ZYC performed the experiments. CLH and SKJ were responsible for data analysis and interpretation. MSC and JCC drafted the manuscript. All authors read and approved the final version of the manuscript. JCC and MSC confirm the authenticity of all the raw data.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

1. Kaur G, Han SJ, Yang I and Crane C: Microglia and central nervous system immunity. *Neurosurg Clin N Am* 21: 43-51, 2010.
2. Spiteri AG, Wishart CL, Pamphlett R, Locatelli G and King NJC: Microglia and monocytes in inflammatory CNS disease: Integrating phenotype and function. *Acta Neuropathol* 143: 179-224, 2022.
3. Muzio L, Viotti A and Martino G: Microglia in neuroinflammation and neurodegeneration: From understanding to therapy. *Front Neurosci* 15: 742065, 2021.
4. Qiu M, Xu E and Zhan L: Epigenetic regulations of microglia/macrophage polarization in ischemic stroke. *Front Mol Neurosci* 14: 697416, 2021.
5. Scheiblich H, Trombly M, Ramirez A and Heneka MT: Neuroimmune Connections in Aging and Neurodegenerative Diseases. *Trends Immunol* 41: 300-312, 2020.
6. Chen MS, Yang KS, Lin WC, Fang CL, Chen HF and Sheu SM: Lipofundin mediates major inhibition of intravenous propofol on phorbol myristate acetate and *Escherichia coli*-induced neutrophil extracellular traps. *Mol Biol Rep* 9: 6517-6529, 2022.
7. Chen MS, Tung YW, Hu CL, Chang HJ, Lin W and Sheu SM: Three lipid emulsions reduce staphylococcus aureus-stimulated phagocytosis in mouse RAW264.7 cells. *Microorganisms* 9: 2479, 2021.
8. Chen MS, Lu PK, Lin WC, Shin HC, Sie SR and Sheu SM: Lipofundin mediates the major inhibition of intravenous propofol in IL-1 $\beta$  secretion and phagocytosis of staphylococcus aureus-infected macrophages. *Lipids* 55: 45-52, 2020.



9. Daniels MJ and Brough D: Unconventional pathways of secretion contribute to inflammation. *Int J Mol Sci* 18: 102, 2017.
10. Turner MD, Nedjai B, Hurst T and Pennington DJ: Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta* 1843: 2563-2582, 2014.
11. Baker HN, Murphy R, Lopez E and Garcia C: Conversion of a capture ELISA to a Luminex xMAP assay using a multiplex antibody screening method. *J Vis Exp* 4084, 2012.
12. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
13. Lo J, Liu CC, Li YS, Lee PY, Liu PL, Wu PC, Lin TC, Chen CS, Chiu CC, Lai YH, *et al*: Punicalagin Attenuates LPS-Induced Inflammation and ROS Production in Microglia by Inhibiting the MAPK/NF- $\kappa$ B Signaling Pathway and NLRP3 Inflammasome Activation. *J Inflamm Res* 15: 5347-5359, 2022.
14. Vinnars E and Wilmore D: Jonathan roads symposium papers. History of parenteral nutrition. *JPEN J Parenter Enteral Nutr* 27: 225-231, 2003.
15. Williams JJ, Mayurasakorn K, Vannucci SJ, Mastropietro C, Bazan NG, Ten VS and Deckelbaum RJ: N-3 fatty acid rich triglyceride emulsions are neuroprotective after cerebral hypoxic-ischemic injury in neonatal mice. *PLoS One* 8: e56233, 2013.
16. Berressem D, Koch K, Franke N, Klein J and Eckert GP: Intravenous treatment with a long-chain omega-3 lipid emulsion provides neuroprotection in a murine model of ischemic stroke - a pilot study. *PLoS One* 11: e0167329, 2016.
17. Tanioka M, Park WK, Park J, Lee JE and Lee BH: Lipid emulsion improves functional recovery in an animal model of stroke. *Int J Mol Sci* 21: 7373, 2020.
18. Zendedel A, Habib P, Dang J, Lammerding L, Hoffmann S, Beyer C and Slowik A: Omega-3 polyunsaturated fatty acids ameliorate neuroinflammation and mitigate ischemic stroke damage through interactions with astrocytes and microglia. *J Neuroimmunol* 278: 200-211, 2015.
19. Chen X, Wu S, Chen C, Xie B, Fang Z, Hu W, Chen J, Fu H and He H: Omega-3 polyunsaturated fatty acid supplementation attenuates microglial-induced inflammation by inhibiting the HMGB1/TLR4/NF- $\kappa$ B pathway following experimental traumatic brain injury. *J Neuroinflammation* 14: 143, 2017.
20. Baazm M, Behrens V, Beyer C, Nikoubashman O and Zendedel A: Regulation of inflammasomes by application of omega-3 polyunsaturated fatty acids in a spinal cord injury model. *Cells* 10: 3147, 2021.
21. Zhao T, Ma D, Mulati A, Zhao B, Liu F and Liu X: Development of astaxanthin-loaded layer-by-layer emulsions: physicochemical properties and improvement of LPS-induced neuroinflammation in mice. *Food Funct* 12: 5333-5350, 2021.
22. Della Giustina A, Goldim MP, Danielski LG, Florentino D, Garbossa L, Joaquim L, Oliveira Junior AN, Mathias K, Fileti ME, Zerbato GF, *et al*: Fish oil-rich lipid emulsion modulates neuroinflammation and prevents long-term cognitive dysfunction after sepsis. *Nutrition* 70: 110417, 2020.
23. Li P and Song C: Potential treatment of Parkinson's disease with omega-3 polyunsaturated fatty acids. *Nutr Neurosci* 25: 180-191, 2022.
24. Wen YT, Huang TL, Huang SP, Chang CH and Tsai RK: Early applications of granulocyte colony-stimulating factor (G-CSF) can stabilize the blood-optic-nerve barrier and ameliorate inflammation in a rat model of anterior ischemic optic neuropathy (rAION). *Dis Model Mech* 9: 1193-1202, 2016.
25. Wen Q, Kong Y, Zhao HY, Zhang YY, Han TT, Wang Y, Xu LP, Zhang XH and Huang XJ: G-CSF-induced macrophage polarization and mobilization may prevent acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 54: 1419-1433, 2019.
26. Rasouli B, Ghahari L, Safari M, Shahroozian E and Naeimi S: Combination therapy of the granulocyte colony stimulating factor and intravenous lipid emulsion protect the hippocampus after global ischemia in rat: focusing on CA1 region. *Metab Brain Dis* 35: 991-997, 2020.
27. Schäbitz WR, Kollmar R, Schwaninger M, Juettler E, Bardutzky J, Schölzke MN, Sommer C and Schwab S: Neuroprotective effect of granulocyte colony-stimulating factor after focal cerebral ischemia. *Stroke* 34: 745-751, 2003.
28. England TJ, Gibson CL and Bath PM: Granulocyte-colony stimulating factor in experimental stroke and its effects on infarct size and functional outcome: A systematic review. *Brain Res Rev* 62: 71-82, 2009.
29. Popa-Wagner A, Stöcker K, Balseanu AT, Rogalewski A, Diederich K, Minnerup J, Margaritescu C and Schäbitz WR: Effects of granulocyte-colony stimulating factor after stroke in aged rats. *Stroke* 41: 1027-1031, 2010.



Copyright © 2024 Chen et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.