European Review for Medical and Pharmacological Sciences 2020; 24: 10181-10193

Menaquinone-7 protects astrocytes by regulating mitochondrial function and inflammatory response under hypoxic conditions

R.-Y. YANG[1], J.-Y. PAN[1], Y. CHEN[1], Y. LI[1], J. WU[1], X.-D. WANG[1,2]

[1]Department of Histology and Embryology, Medical College, Nantong University, Nantong, China
[2]Key Laboratory for Neuroregeneration of Ministry of Education and Co-innovation Center for Neuroregeneration of Jiangsu Province, Nantong University, Nantong, China

Riyun Yang and Jingying Pan contributed equally to this work

Abstract. – OBJECTIVE: Astrocytes play a key role in hypoxic brain injury. The aim of our research was to determine the effects of menaquinone-7 (MK-7), a subtype of vitamin K2 (VK2), on astrocytes during hypoxia and its potential mechanisms.

MATERIALS AND METHODS: Astrocytes from the palliums of newborn Sprague Dawley rats were cultured. An astrocyte-hypoxia model was established using a hypoxia workstation. Cell Counting Kit-8 (CCK-8) and BrdU assays were used to determine the effects of MK-7 on hypoxic astrocytes. 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) or dihydroethidium (DHE) assays were conducted to detect the levels of reactive oxygen species (ROS). An ATP assay was used to measure intracellular ATP production. The levels of proinflammatory cytokines and chemokines containing interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), CC-chemokine ligand 2 (CCL2), and CXC-chemokine ligand 10 (CXCL10), as well as vitamin K-dependent protein growth arrest-specific 6 (Gas6), were determined in hypoxia-induced astrocytes, in the presence or absence of MK-7 pretreatment. Small interfering RNA (siRNA) was used to knockdown Gas6 expression to determine its role in hypoxic astrocytes pretreated with MK-7.

RESULTS: Hypoxia reduced astrocyte viability and proliferation significantly; however, when pretreated with MK-7, these conditions remarkably increased. MK-7 also inhibited hypoxia-induced ROS production and enhanced ATP generation of hypoxic astrocytes. Pretreatment with MK-7 effectively reduced the expression of IL-6, TNF-α, CCL2, and CXCL10 but enhanced the expression of Gas6 in hypoxic astrocytes. Gas6 inhibition markedly attenuated the decline in MK-7-induced ROS generation and IL-6 expression, and weakened MK-7-induced cell viability and ATP production in hypoxic astrocytes.

CONCLUSIONS: Our study is the first to confirm that MK-7 can protect astrocytes from hypoxia-induced cytotoxicity, possibly by inhibiting mitochondrial dysfunction and the expression of proinflammatory cytokines. Gas6 may also participate in these protective effects.

Key Words:
Astrocytes, Menaquinone-7, Proliferation, Hypoxia, Mitochondria, Inflammation, Gas6.

Introduction

Astrocytes, the chief glial cell type within the central nervous system (CNS), have many functions, such as maintaining homeostasis of the brain. However, when hypoxia/ischemia exceeds the body's tolerance levels, the astrocytes are damaged, which causes a loss to normal CNS functions, generates inflammatory cytokines, and could promote the death of adjacent neurons and increase damage to the brain[1-3]. Therefore, protecting damaged astrocytes can provide a promising strategy for preserving neuronal integrity and function, and for protecting brain function in hypoxic/ischemic brain injury in a direct and indirect manner. Hypoxia leads to the excessive production of reactive oxygen species (ROS) in mitochondria and sequentially gives rise to oxidative stress[4,5], which can result in damage to mitochondria[6]. Dysfunctional mitochondria contribute to inflammatory responses[7,8]. Inflammation and oxidative stress are closely connected and can lead to cell death. Therefore, protecting mitochondrial function and mitigating inflammation could have vital effects on hypoxic astrocytes and

Corresponding Authors: Xiaodong Wang, MM; e-mail: wxdzw@ntu.edu.cn
Riyun Yang; e-mail: yangriyunyx@163.com

provide new insights into hypoxic/ischemic brain injury.

Vitamin K was initially found for its function in blood coagulation. The roles of vitamin K in the nervous system have attracted wide attention recently. Menaquinone (vitamin K2, VK2) is one of the biologically active forms of vitamin K[9]. VK2 has 14 isoforms and are classified by the number of their isoprenoid units in the side chain[10]. Menaquinone-7 (MK-7), a member of the VK2 family, is the highest bioactive form, has the longest half-life[11], and is considered to be the superior form of VK2 for optimum bioavailability[12]. VK2 is an essential element in the synthesis and metabolism of sphingolipids, which is abundant in the cell membranes of the brain. Sphingolipids participate in vital cellular events, including proliferation, differentiation, senescence, and intercellular interactions[13]. Thus, VK2 have the potential to affect the function of brain. It could promote neuronal cell survival and protect neurons and oligodendrocyte precursors from damage by oxidative stress[14-16]. VK2 is a necessary part of the electron transport pathway in mitochondria[17,18], and MK-7 can inhibit inflammatory reactions by downregulating IL-6[19] and tumor necrosis factor-alpha (TNF-α)[20]. However, the effect of MK-7 on hypoxic astrocytes has never been reported even if it is the most effective subtype of VK2.

Based on the study and analysis of the citations, we hypothesized that MK-7 may have a potential pharmacological effect on hypoxia-induced cytotoxicity in astrocytes. The primary aim of the research was to detect the effects of MK-7 on cell viability and proliferation, oxidative stress, mitochondrial function, and inflammatory response in hypoxic astrocytes and its underlying mechanisms.

Materials and Methods

Primary Astrocyte Culture

Primary astrocytes were taken from the palliums of 1-y-old to 2-y-old Sprague-Dawley (SD) rats (Animal Laboratory Center, Nantong University, Nantong, China) and cultured, as previously described[21]. All protocols with animals were approved by Nantong University Animal Ethics Committee, and the studies were conducted according to the management regulations and methods of experimental animals. Neonatal rats were submerged in 75% alcohol for 5 min and washed with Hank's balanced salt solution (Gibco, Grand Island, NY, USA). The heads of rat pups were excised, and the cerebral cortices were isolated on ice. After the meninges and large blood vessels were removed, the dissociated palliums were minced and digested using 0.125% trypsin-EDTA (Gibco, Grand Island, NY, USA) at 37°C for 40 min. The tissue suspension was pipetted to scatter the cells, and then, filtered using a 100-μM pore membrane (Merck KGaA, Darmstadt, Germany). The cell suspension was centrifuged at 1200 rpm for 4 min, after which the pellet obtained was resuspended in DMEM/F12 (Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Beyotime, Shanghai, China). Dissociated cells were added to a T75 culture flask at a density of 1.5×10^6 cells/mL and incubated at 37°C and 5% CO_2/95% air. The culture medium was replaced every 2 d. When the cells achieved confluence, any microglia cells were eliminated by shaking at 280 rpm for 18 h. Before the further study, the astrocytes were passaged twice.

The astrocytes were divided into the pretreatment group and no pretreatment group. In the pretreatment group, the astrocytes were pretreated for 30 h with MK-7 (MenaQ7, NattoPharma ASA, Høvik, Norway) concentrations (10, 50, 100, and 150 μM) with 10% FBS in DMEM/F12. They were then maintained under either normoxic (5% CO_2/95% air, 37°C) or hypoxic (5% CO_2/95% N2, 37°C) conditions for different time intervals. The culture medium was changed to glucose-free Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) without FBS before incubating the astrocytes under hypoxic conditions. Hypoxia was induced by incubating the astrocytes using the hypoxic workstation (Don Whitley Scientific, West Yorkshire, UK).

Cell Counting Kit-8 Assay

Astrocyte viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions. Astrocytes were plated onto 96-well culture plates at a density of 6×10^3 cells/well. After being pretreated with 10, 50, 100, and 150 μM MK-7 for 30 h, astrocytes were maintained under hypoxic conditions for 12 h. Later, 15 μL CCK-8 solutions was added to each well, and the astrocytes were maintained at 37°C for an additional 2 h. Cell absorbance was detected at 450 nm using a multifunctional microplate reader (Biotek, Winooski, VT, USA).

BrdU Assay

The BrdU assay was conducted using the BrdU Cell Proliferation ELISA Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The astrocytes were plated onto a black 96-well plate and were pretreated with 10, 50, 100, and 150 μM MK-7, and then, cultured under hypoxic conditions for 12 h. After then, 10 μM BrdU was added and the cells were cultured for another 18 h. After fixation, the astrocytes were maintained with 100 μL/well anti-BrdU-POD working solution for 100 min at 25°C. After incubating for 10 min with substrate solution, the light emission of the cells was measured using a multifunction microplate reader.

Detection of Reactive Oxygen Species

Intracellular ROS was first determined using a DCFDA Cellular ROS Assay Kit (Abcam, Cambridge, MA, USA). DCFDA is a fluorescent probe that can measure ROS, such as hydrogen peroxide, peroxynitrite, and peroxyl radical[22]. The cells were plated onto a 96-well plate and cultured under normoxic or hypoxic conditions at different time intervals (4, 6, 8 and 12 h) in the presence or absence of pretreatment with multiple MK-7 (10, 50, 100, and 150 μM). Afterward, the astrocytes were stained with DCFDA for 45 min. Fluorescence from each well was detected using a multifunction microplate reader with an excitation/emission at 485 nm/535 nm.

ROS was detected using a confocal microscope (Leica, Wetzlar, Germany). DHE (Sigma-Aldrich, St Louis, MO, USA) is a red fluorescent probe that is generally applied as a superoxide probe[23]. The astrocytes were incubated in the presence or absence of pretreatment for 30 h with 150 μM MK-7 and maintained under normoxic or hypoxic conditions for 12 h. Subsequently, the cells were maintained with 50 μM DHE for 30 min at 37°C in a dark humidified chamber, and then, washed four times with cold PBS. The cell images were captured immediately under a confocal microscope at 488 nm excitation and 600 nm emission wavelength.

Measuring ATP Levels

The amount of ATP under each experimental condition was assessed using a multifunction microplate reader utilizing an ATP assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. The astrocytes were pretreated with 10, 50, 100, or 150 μM MK-7 for 30 h followed by stimulation with hypoxia for 12 h. The cells were harvested and resuspended in 100 μL ATP assay buffer. The samples were centrifuged in 4°C at 13000 rpm for 5 min to collect the supernatant, after which sample and standards were added to a 96-well plate. The reaction mixture was then added to each well and maintained for 30 min. A multifunctional microplate reader was used to detect the absorbance of the samples and standards at 570 nm.

Quantitative Real-time Polymerase Chain Reaction

Total RNA from the cultured astrocytes under normoxic or hypoxic conditions and with or without pretreatment of MK-7 was isolated using TRI reagent (Sigma-Aldrich, St Louis, MO, USA). RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted using the Bio-Rad CFX96 Real-Time PCR System with SYBR Green PCR Kit (Qiagen, Duesseldorf, Germany). Primers were designed using Primer 5 software (ver. 1.0). The PCR primers were listed below (Table I). Relative expression of each gene was normalized to β-actin.

Western Blotting

Proteins were extracted from the astrocytes in each experimental group using a cell lysis buffer (Abcam, Cambridge, MA, USA) containing 1 mM PMSF (Abcam, Cambridge, MA, USA). The protein concentration was detected using the bicinchoninic acid assay (BCA) Protein Assay Kit (Abcam, Cambridge, MA, USA). Equivalent amounts of protein from each experimental group were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). The membrane was maintained with specific primary antibodies overnight at 4°C before blocking with 5% fat-free milk. After washing three times with Tris-Buffered Saline and Tween-20 (TBST), the membrane was maintained with the corresponding horseradish peroxidase-conjugated secondary antibody for 2 h. The blots were visualized using chemiluminescence substrate (Merck Millipore, Burlington, MA, USA) with ChemiDoc (BioRad, Hercules, CA, USA), and quantified using densitometry with Quantity One software. The specific primary antibodies were used as follows: anti-Gas6 (1:1000,

Table I. Primers for quantitative RT-PCR (F = forward primer, R = reverse primer, 5′-3′).

Gene	Sequence
IL-6	F: TCCTACCCCAACTTCCAATGCTC
	R: TTGGATGGTCTTGGTCCTTAGCC
TNF-α	F: AAATGGGCTCCCTCTCATCAGTTC
	R: TCTGCTTGGTGGTTTGCTACGAC
Ccl2	F: AATGAGTCGGCTGGAGAA
	R: GCTTGAGGTGGTTGTGGA
Cxcl10	F: GGGCCATAGGAAAACTTGAAATC
	R: CATTGTGGCAATGATCTCAACAT
Gas6	F: CCCCCGTGATTAGACTACGC
	R: GATCCAGGTGCTATCCGAGC
β-actin	F: CTAAGGCCAACCGTGAAA
	R: CTCGAAGTCTAGGGCAAC

Abcam, Cambridge, MA, USA) and anti-β-actin (1:3000, Beyotime, Shanghai, China).

Transfection of siRNA

Gas6 siRNA was designed and synthesized by OBiO Biotechnologies (Shanghai, China). The siRNA sequences of Gas6 were as follows: si-1 forward CAAGAGCUGCCAAGAUAUATT, reverse UAUAUCUUGGCAGCUCUUGTT; si-2 forward GCAAACGAUCUCUGUGGA

ATT, reverse UUCCACAGAGAUCGUUUGCTT. After confirming the knockdown efficiency of these two siRNAs using RT-PCR, the more efficient siRNA was used to silence Gas6 expression in the astrocytes. Gas6-siRNA or a negative scrambled control siRNA was transfected into the astrocytes using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA, USA). After transfecting for 48 h, the astrocytes were collected and used for further analyses.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software (ver. 6.01). Significance tests were conducted using one-way analysis of variance. The data were presented as the means ± standard deviation (SD). $p<0.05$ indicated statistical significance.

Results

MK-7 Reversed the Attenuated Astrocyte Viability and Proliferation Induced by Hypoxia in a Dose-Dependent Manner

Rat primary astrocytes were stimulated by hypoxia before the CCK-8 and BrdU assays were conducted. As revealed in Figure 1A and B, there was a significant decline in cell viability and proliferation under hypoxic conditions over all time intervals. Hypoxia for 12 h induced the most serious damage in the astrocytes. Therefore, we chose that time frame for further experiments. For the purpose of assessing the effect of MK-7 on hypoxic astrocytes, the cells were pretreated with a range of MK-7 concentrations before being cultured under hypoxic conditions. The impact of MK-7 on astrocyte viability and proliferation was barely detectable when it was applied under normoxic condition (Figure 1C and 1D, $p>0.05$). Notably, the attenuated cell viability and proliferation induced by hypoxia was reversed in the presence of MK-7 (Figure 1E and 1F, $p<0.05$), and the effects was the best after pretreatment with 150 μM MK-7. Therefore, we chose 150 μM MK-7 for further experiments. Astrocyte viability and proliferation were enhanced in parallel with increases in MK-7 concentration under hypoxic conditions.

MK-7 Decreased the Morphological Alteration of Hypoxic Astrocytes

To confirm the role of MK-7 in promoting astrocyte viability and proliferation under hypoxic conditions, morphological alterations in astrocytes were observed using an inverted microscope. As shown in Figure 1G-H, astrocytes under normoxic conditions were applanate and polygonal. However, pretreatment with MK-7 did not cause these apparent morphological alterations in these astrocytes. Following hypoxia treatment for 12 h, some cells shredded and floated, and some were rounded and swollen. Nevertheless, the morphological alterations in astrocytes pretreated with MK-7 were reduced and the number of adherent astrocytes significantly increased under hypoxic conditions.

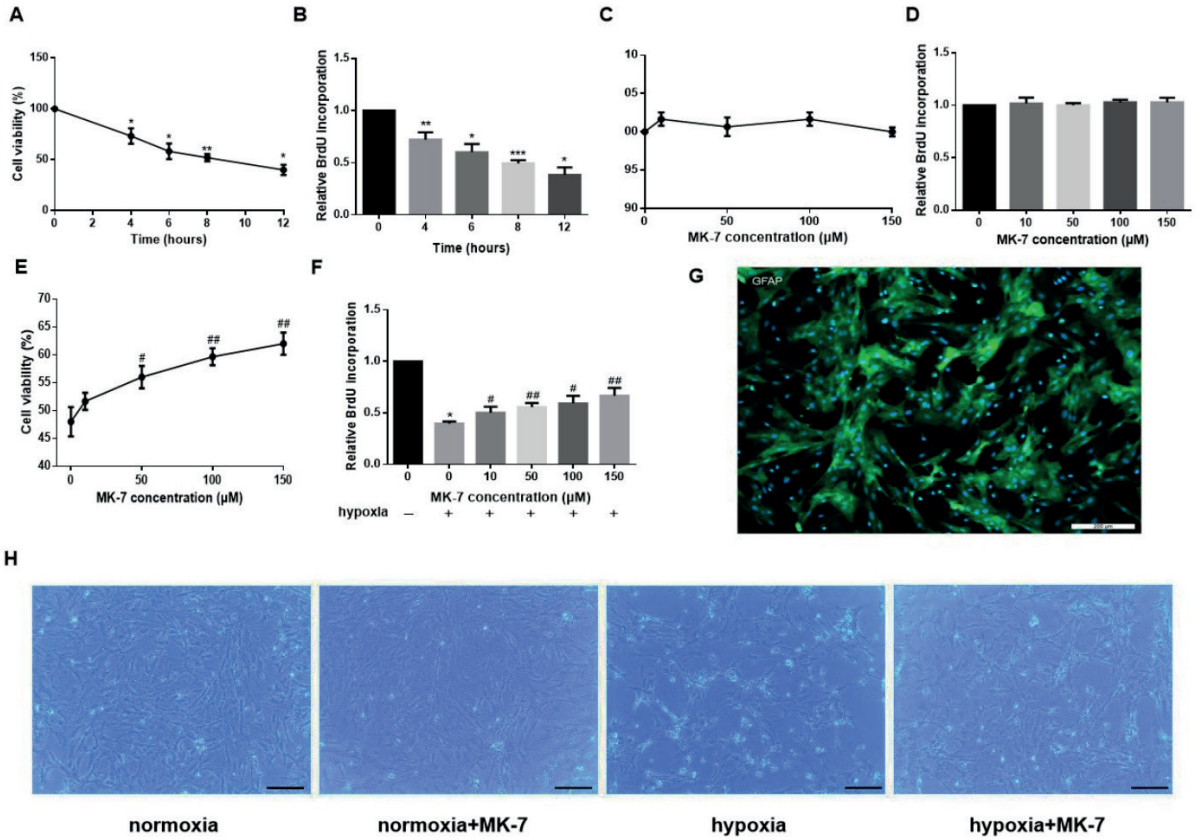

Figure 1. MK-7 promoted the astrocyte viability and proliferation under hypoxia. The primary astrocytes were cultured under hypoxic conditions for 4, 6, 8, and 12 h, and cell viability and proliferation was measured using the CCK-8 assay **(A)** or BrdU assay **(B)**. The astrocytes were pretreated with increasing concentrations of MK-7 for 72 h under normoxic conditions, followed by analysis of cell viabilty **(C)** and proliferation **(D)**. CCK-8 assay **(E)** or BrdU assay **(F)** was used to detect cell viability and proliferation in the MK-7 pretreated astrocytes under hypoxic conditions for 12 h. **G,** Immunofluorescence of glial fibrillary acidic protein in the primary astrocytes (scale bar: 200 μm). **H,** Representative images of the morphological alterations of the astrocytes cultured under normoxic or hypoxic conditions for 12 h in the presence or absence of 30 h pretreatment with 150 μM MK-7(scale bar: 200 μm). Notes: *p<0.05, **p<0.01, ***p<0.001 compared to the normoxia group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01 compared to cells treated with hypoxia alone; n=6 independent experiments.

MK-7 Alleviated the Oxidative Stress Induced by Hypoxia in Astrocytes

To detect whether hypoxia-induced attenuated astrocyte viability and proliferation are relevant to intracellular oxidative stress, the ROS levels generated by the astrocytes was measured under hypoxic conditions in the presence or absence of MK-7 pretreatment. The results showed that hypoxia stimulation led to a remarkable enhancement in ROS generation in the astrocytes, but that generation was not modified in response to pretreatment with MK-7 under normoxic conditions. After MK-7 pretreatment, the level of ROS in hypoxia-stimulated astrocytes was significantly lower than in hypoxic cells not pretreated (Figure 2A and 2C, p<0.05). In addition, the prohibitive function of MK-7 on ROS generation in hypoxic astrocytes was dose dependent (Figure 2B, p<0.05). These results suggested that MK-7 could protect astrocytes from oxidative stress by decreasing the intracellular generation of ROS, and then, promote the viability and proliferation of astrocytes under hypoxic conditions.

MK-7 Eased Oxidative Stress-Induced Mitochondria Injury in Astrocytes Under Hypoxic Conditions

Mitochondria are the primary cytoplasmic organelles for the generation of ROS, which play significant roles in supporting the normal function of cells; however, excessive ROS can damage mitochondria[6]. To explore whether MK-7 can regulate mitochondrial function in hypoxic astrocytes, the ATP levels produced by mitochondria

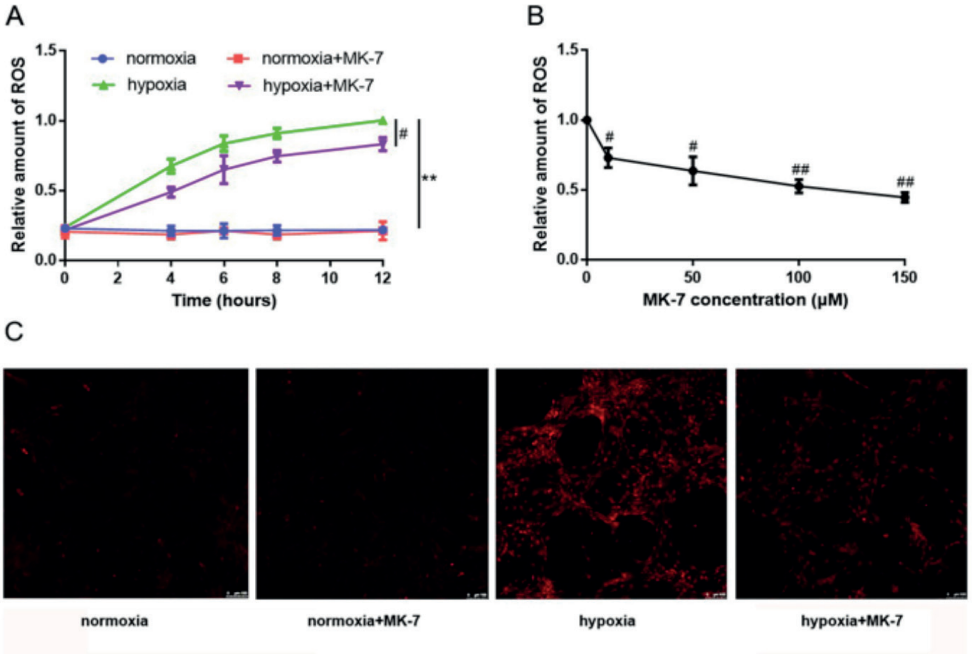

Figure 2. MK-7 mitigated hypoxia-caused oxidative damage in the astrocytes. **A,** The astrocytes were cultured under normoxic or hypoxic conditions for 4, 6, 8, or 12 h in the presence or absence of 30 h pretreatment with 10 μM MK-7, and then reactive oxygen species (ROS) production was analyzed using the DCFDA Cellular ROS Assay Kit. **B,** DCFDA Cellular ROS Assay Kit was used to detect ROS production in the pretreated astrocytes under hypoxic conditions for 12 h. **C,** ROS immunofluorescence in the both pretreated and non-pretreated astrocytes was measured using dihydroethidium staining (scale bar: 100 μm). Notes: $^{**}p<0.01$ compared to the normoxia alone group, and $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with cells treated with hypoxia alone; n=3 independent experiments.

were assessed in the pretreated astrocytes. As shown in Figure 3A and 3C, hypoxia significantly inhibited ATP production. However, MK-7 reversed it to a certain degree in the astrocytes in a dose dependent manner. Meanwhile, MK-7 barely influenced the ATP levels in astrocytes under normoxic conditions (Figure 3B, $p<0.05$). The above results demonstrated that the protective effect of MK-7 might be relevant to regulating the function of mitochondria in hypoxic astrocytes.

MK-7 Attenuated the Inflammatory Response Induced by Hypoxia in Astrocytes

Hypoxia can induce inflammatory changes, and oxidative stress is a vital mechanism of the inflammatory reactions[24]. Meanwhile, mitochondria plays critical roles in the occurrence and development of inflammation[25]. To determine whether MK-7 participates in regulating the inflammatory response of hypoxic astrocytes, the astrocytes were pretreated with various concentrations of MK-7 and then maintained under hypoxic conditions. The results revealed that hypoxia strongly enhanced the level of proinflammatory cytokines and chemokines, yet MK-7 pretreatment dose-dependently reduced the upregulation of IL-6, TNF-a, CC-chemokine ligand 2 (CCL2), and CXC-chemokine ligand 10 (CXCL10) (Figure 4, $p<0.05$). These data revealed that hypoxia-induced astrocytic inflammatory response was decreased by MK-7 pretreatment and thus protected the astrocytes.

MK-7 Reduced Mitochondria Damage and Inflammatory Response by Promoting Gas6 Expression

Gas6 is a vitamin K-dependent protein that participates in several cellular processes that affect cell survival, proliferation, adhesion, and chemotaxis[26]. Gas6 can also attenuate inflammation[27] and impacts mitochondrial function[28]. To further research the potential mechanism by which MK-7 protects astrocytes under hypoxia, the Gas6 level was analyzed in the presence or absence of MK-7 pretreatment. As shown in Figure 5A and 5B, hypoxia remarkably decreased the mRNA and protein expressions of

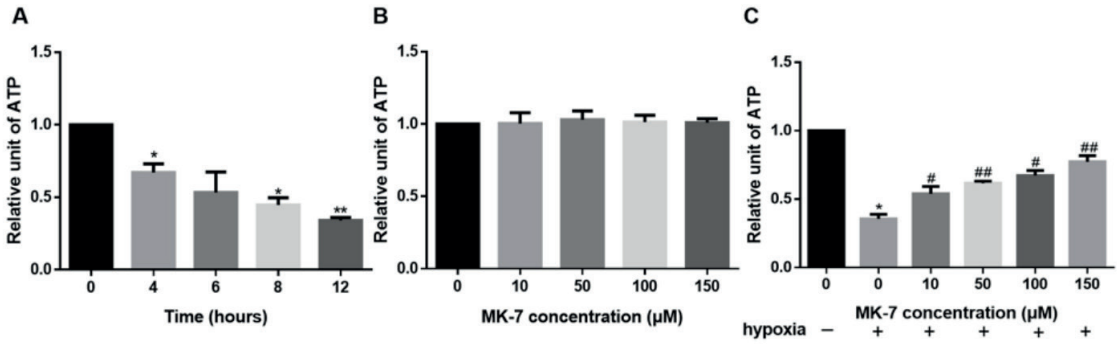

Figure 3. MK-7 protection of mitochondria in hypoxic astrocytes. **A**, Levels of ATP in the astrocytes cultured under hypoxia for 4, 6, 8, and 12 h. **B,** The astrocytes pretreated with increasing concentration of MK-7 72 h under normoxic conditions. **C,** The astrocytes pretreated with 10, 50, 100, or 150 μM MK-7 for 30 h and cultured under hypoxic conditions for another 12 h. Notes: Data were expressed as a percent of level of ATP in normoxic astrocytes without MK-7 pretreatment. *p<0.05, **p<0.01 compared to the normoxia alone group, and $^{\#}p$<0.05, $^{\#\#}p$<0.01 compared with cells treated with hypoxia alone; n=3 independent experiments.

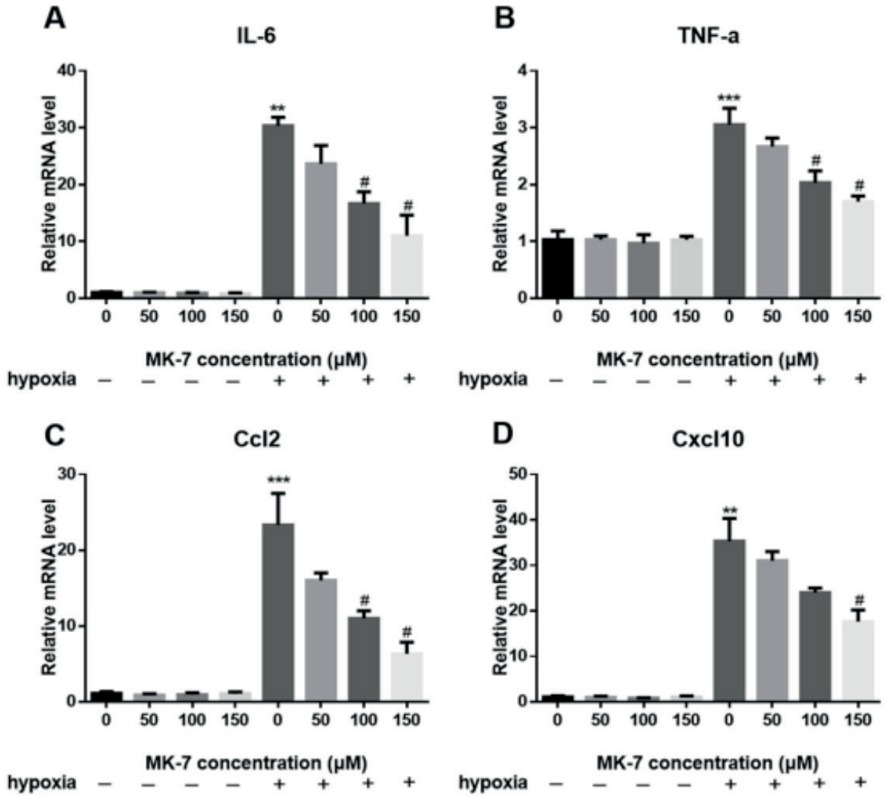

Figure 4. MK-7 pretreatment attenuated proinflammatory cytokine and chemokine expression in the hypoxic astrocytes. The astrocytes pretreated with 50, 100, or 150 μM MK-7 for 30 h and cultured under normoxic or hypoxic conditions for another 12 h, then IL-6 **(A)**, TNF-a **(B)**, Ccl2 **(C)**, and Cxcl10 **(D)** levels detected using quantitative RT-PCR analysis. Notes: **p<0.01, ***p<0.001 compared to the normoxia alone group, and $^{\#}p$<0.05 compared with cells treated with hypoxia alone; n=3 independent experiments.

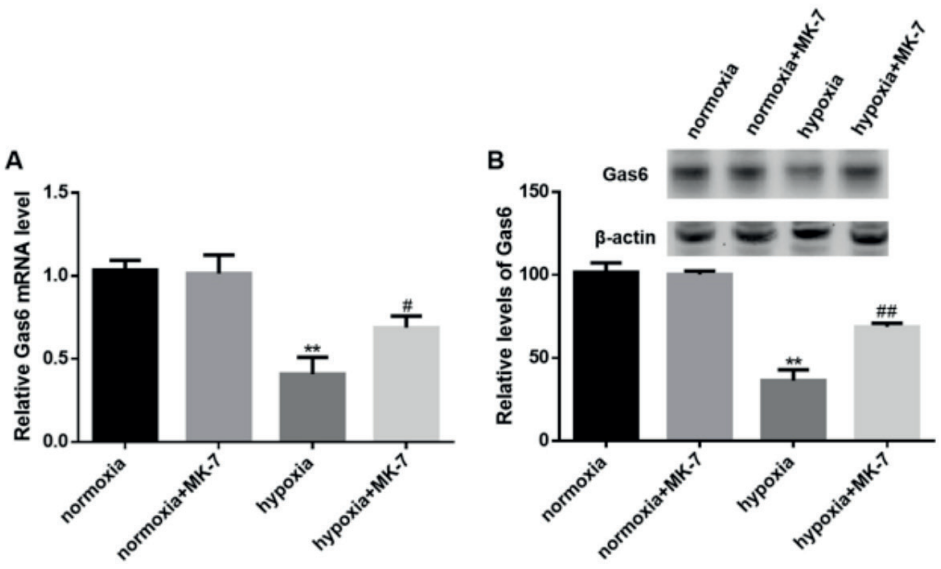

Figure 5. MK-7 promoted Gas6 expression in the hypoxic astrocytes. The astrocytes were cultured under normoxic or hypoxic conditions for 12 h in the presence or absence of 30 h pretreatment with 150 μM MK-7. **A,** Gas6 mRNA levels. **B,** Protein levels of Gas6. Notes: β-actin was utilized as an internal control. $^{**}p<0.01$ compared to the normoxia alone group, and $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with cells treated with hypoxia alone; n=3 independent experiments.

Gas6. However, MK-7 pretreatment significantly reversed the Gas6 levels in the astrocytes. The results indicated that Gas6 might be involved in the protective properties of MK-7 on hypoxic astrocytes. To further show this, siRNAs were used to silence Gas6 expression and the more-efficient siRNA was used in the subsequent experiments (Figure 6A and 6B, $p<0.05$). As shown in Figure 6C-F, the astrocyte viability in MK-7 in the si-Gas6 group was markedly decreased compared with that in the MK-7 group, all treated under hypoxic conditions. The results suggested that the knockdown of Gas6 could inhibit the protective property of MK-7 on hypoxic astrocytes. The ROS, IL-6, and ATP levels in the MK-7 pretreated hypoxic astrocytes were measured under si-Gas6 conditions. The results indicated that MK-7 treatment recovered the levels of ROS, IL-6, and ATP in astrocytes under hypoxic conditions, but that Gas6 silencing reversed the effect. Together, the above results demonstrated that the knockdown of Gas6 could inhibit the protective property of MK-7 on hypoxic astrocytes and that the protective effect of MK-7 was connected to the regulation Gas6 expression.

Discussion

Astrocytes, the widely distributed cell type in the brain, can generate trophic factors, synthesize and secrete neurotransmitters, scavenge toxins, as well as maintain homeostasis in the brain[29,30]. The pathophysiological role of astrocytes in hypoxic/ischemic brain injury has recently attracted much attention. In the process of hypoxia/ischemia, astrocytes may be more vulnerable than neurons and promote secondary neuronal death[31]. Astrocytes serve as defenders of the brain[32]; therefore, searching for efficacious therapeutic drugs for injured astrocytes would provide valuable contributions to hypoxic/ischemic brain injury.

Hypoxia induces reductive carboxylation, which results in increases in ROS generation[33] and favours oxidative stress[5]. During hypoxia, the over-generation of ROS causes mitochondrial dysfunction[6], which is the main source of ROS[34]. Mitochondrial failure from impaired mitochondrial proteins and membranes inhibits its ability to generate normal levels of ATP[35], which contributes to the inflammatory response[7,8]. Hypoxia leads to alterations in cellular bioenergetics, which promotes inflammation[5]. Inflammation

and oxidative stress are interdependent and interconnected processes, and the pathways leading to cell death are closely connected[36]. In our research, we demonstrated that hypoxia-induced oxidative stress from excessive ROS resulted in mitochondrial dysfunction and triggered inflammatory responses as well as cascade-dependent cell injury in astrocytes.

Vitamin K is initially known for its function as a cofactor for γ-glutamyl carboxylase (GGCX), which activates the coagulation factors II, VII, IX, and X[9]. A lack of vitamin K results in bleeding. VK2 is one of active forms of vitamin K. VK2 is a range of chemical compounds known as menaquinones, which has 14 isomers (MK1-MK14) with different isoprenoid units. MK-7 is the most biologically active form and has the greatest bioavailability in the VK2 family[12]. Meanwhile, MK-7 has a longer half-life (3 d) than MK-4 (1-2 h) in the blood[37,38]. Therefore, MK-7 can act as a time-released source of VK2. MK-7 has been used as a therapeutic nutrient in many countries for the prevention or reversal of osteoporosis and angiocardiopathy. VK2 can induce 3-ketodihydrosphingosine (3-KDS) synthase, which take part in the initial step of sphingolipids synthesis[39]. Sphingolipids are important components of cell membranes. In brain, the cells have particularly high concentrations of sphingolipids. Apart from their structural action, sphingolipids are involved in key cellular events, such as proliferation, differentiation, senescence, and intercellular interactions[39,40]. Koshihara et al[41] have also shown that VK2 can promote osteoblast proliferation and differentiation to improve cellular function. Meanwhile, VK2 inhibits Fas-induced apoptosis of osteoblasts in a dose-dependent manner[42]. VK2 has survival-promoting functions on neurons that were isolated from the embryonic cortex, hippocampus, and striatum[15]. Similarly,

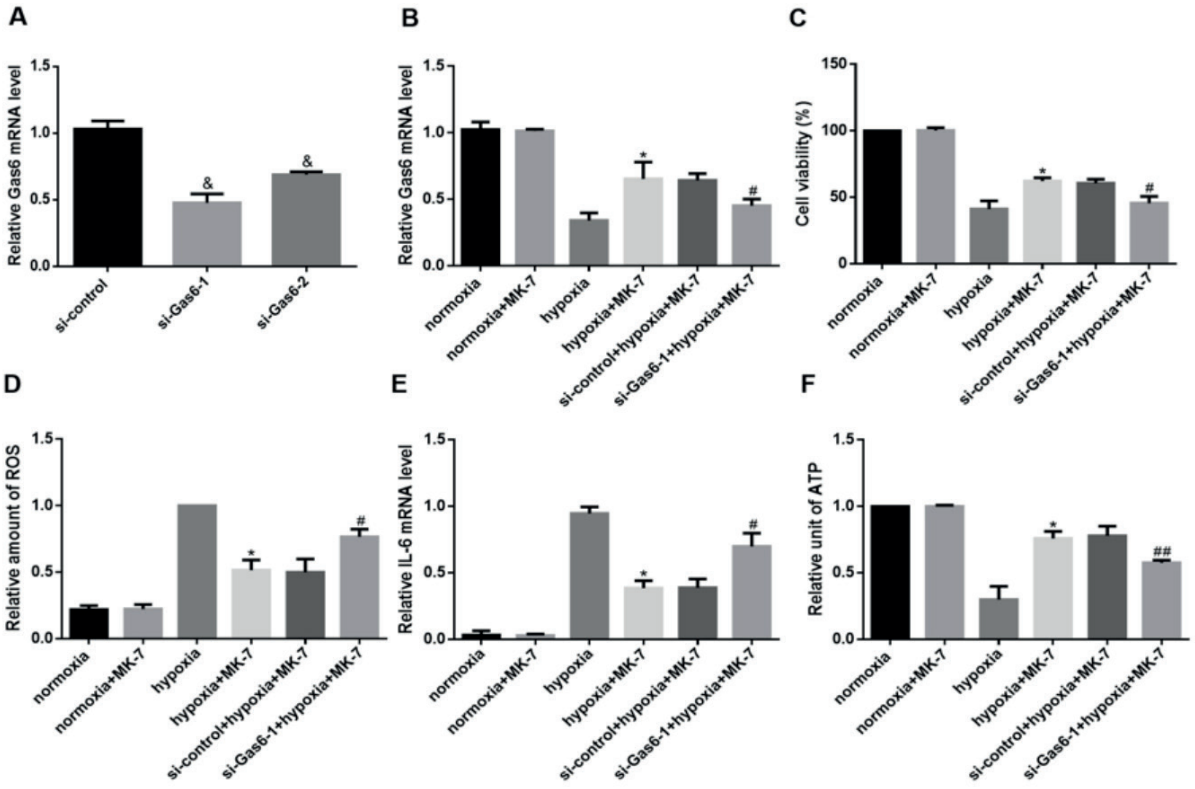

Figure 6. Knockdown of Gas6 reduced the protective influence of MK-7 in the hypoxic astrocytes. **A,** Gas6 mRNA expression in astrocytes transfected with si-Gas6. **B,** Levels of Gas6 mRNA after pretreatment of 150 μM MK-7 in astrocytes with si-Gas6 under hypoxic conditions for 12 h. **C,** Effects of Gas6 siRNA on the increases in MK-7-induced cell viability in astrocytes under hypoxic conditions. Effects of Gas6 siRNA on the decrease in MK-7-induced reactive oxygen species (ROS) generation **(D)** and IL-6 expression **(E)** in the hypoxic astrocytes. **F,** Effects of Gas6 siRNA on the increase in MK-7-induced ATP production in the hypoxic astrocytes. Notes: $^{\&}p<0.01$ compared to the si-control group, $^{*}p<0.01$ compared to the hypoxia alone group, and $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to the hypoxia+MK-7 group.

the present study demonstrated that MK-7 could increase the viability and proliferation of hypoxic astrocytes. Meanwhile, MK-7 could normalize the morphological characteristics of astrocytes under hypoxic conditions.

Li et al[16] have shown that VK2 can protect developing oligodendrocytes and immature fetal cortical neurons from glutathione depletion-induced cell death by inhibiting oxidative stress. In arachidonic acid-mediated oxidative damage of developing oligodendrocytes, VK2 can remarkably block 12-lipoxygenase activation and avoid ROS accumulation, thereby reducing cell mortality[43]. In another methylmercury-induced cell oxidative stress model, VK2 significantly reduced neuron death[44]. VK2 is needed within the electron-transport chain in the mitochondria[17] and acts as a mitochondrial electron carrier to protect impaired mitochondrial function and thus maintain normal ATP production[18]. Our studies have detected that MK-7 can partially reduce hypoxia-induced oxidative stress by balancing ROS production and reverse mitochondrial dysfunction by optimizing ATP generation. Taken together, these data revealed that MK-7 exhibited protective effects under antioxidative stress generally by restoring the function of mitochondria in hypoxic astrocytes.

It has also been shown the protective roles of VK2 in inflammation. VK2 can reverse the increase in inflammatory cytokine levels caused by lipopolysaccharides (LPSs) in cultured microglial[45] and macrophages[46] by inhibiting the nuclear factor-kappaB (NF-κB) signaling pathway. Meanwhile, VK2 has been revealed to restrict the generation of IL-6 in cultured fibroblasts[47]. In human monocyte-derived macrophages, MK-7 decreases the levels of IL-1a, IL-1β, and TNF-a mediated by toll-like receptor agonists[20]. *In vivo*, VK2 can considerably ameliorate inflammation in rat models of autoimmune encephalomyelitis[48]. Furthermore, one clinical research has confirmed that MK-7 can act as an efficient pharmacological treatment for rheumatoid arthritis[11]. Our findings illustrated that MK-7 treatment could attenuate an inflammatory response by decreasing the proinflammatory cytokines and chemokines after hypoxia stimulation in astrocytes. As indicated above, MK-7 can ameliorate hypoxia-induced oxidative stress, mitochondrial damage, and inflammatory reactions to promote astrocyte viability and proliferation. Thus, MK-7 provides a hopeful protective treatment in hypoxic astrocytes and may be helpful in hypoxic/ischemic brain injury.

Gas6 is a vitamin K-dependent protein that is widely expressed and plays important roles in CNS[49]. Gas6 has been verified to participate in cell survival, proliferation, adhesion, and cell growth of neurons and glia cells[39]. Gas6 can activate mitogen-activated protein kinase (MAPK) and the subsequent recruitment of extracellular signal-regulated kinase (ERK), where Gas6 activation of phosphatidylinositol 3-kinase (PI3-K) results in the phosphorylation of serine/threonine protein kinase (Akt). A prosurvival effect of Gas6 has been observed on hippocampal neurons through the activation of MAPK and PI3-K signaling pathways[50]. Allen et al[51] have also shown that Gas6 has a prosurvival function on gonadotropin-releasing hormone neurons, and oligodendrocytes[52] by activating the PI3-K signaling pathway. Gas6 can reverse amyloid β protein-mediated apoptosis of cortical neurons[53]. In metaphase II, Gas6-depleted oocytes exhibit excessive mitochondrial activation, which leads to excessive ROS production[54]. Gas6 regulates mitochondrial autophagy and protects mitochondrial function by regulating the expression of mitochondrial autophagy-related genes[55]. In addition to the above functions, Gas6 regulates the inflammation reactions of microglia by inhibiting the microglia phenotype after LPS stimulation, which demonstrates its anti-inflammatory function[56]. Meanwhile, in LPS-induced microglia, Gas6 can decrease the levels of proinflammatory IL-1β and the mediator nitric oxide synthase (iNOS)[57]. However, whether MK-7 treatment is related to Gas6 in hypoxic astrocytes remains unclear. Our results have indicated that hypoxia leads to a decrease in Gas6 expression, which markedly increased after the astrocytes were pretreated with MK-7. Gas6 was tightly connected to the MK-7-induced protective effects that were somewhat reversed by Gas6 siRNA in astrocytes under hypoxic conditions.

To our knowledge, this work is the first report to utilize MK-7 for the treatment of hypoxia-induced astrocytes. The study verified that MK-7 decreased hypoxia-initiated cytotoxicity within astrocytes, which was closely associated with the pathogenesis of hypoxic/ischemic brain injury. These results suggested a new case for the further development of therapeutic strategies.

Conclusions

Our results suggest that MK-7 efficaciously ameliorates hypoxia-induced cell damage by decreasing ROS-induced oxidative damage and

mitochondrial injury in astrocytes. MK-7 also attenuates the hypoxia-initiated astrocytic inflammatory response and exerts cytoprotection possibly through Gas6. This work promotes the understanding of MK-7 on hypoxic/ischemic brain injury pathophysiological changes at the cellular level and provides a new potential therapeutic strategy for hypoxic/ischemic brain injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (81971763), Natural Science Research of Jiangsu Higher Education Institutions of China (19KJB310012), Nantong Civic Science And Technology Projects of China (JC2018054, JC2018062, JC2019072) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

1) Belov Kirdajova D, Kriska J, Tureckova J, Anderova M. Ischemia-triggered glutamate excitotoxicity from the perspective of glial cells. Front Cell Neurosci 2020; 14: 51.
2) Rossi DJ, Brady JD, Mohr C. Astrocyte metabolism and signaling during brain ischemia. Nat Neurosci 2007; 10: 1377-1386.
3) Gouix E, Buisson A, Nieoullon A, Kerkerian-Le Goff L, Tauskela JS, Blondeau N, Had-Aissouni L. Oxygen glucose deprivation-induced astrocyte dysfunction provokes neuronal death through oxidative stress. Pharmacol Res 2014; 87: 8-17.
4) Jiang L, Shestov AA, Swain P, Yang C, Parker SJ, Wang QA, Terada LS, Adams ND, McCabe MT, Pietrak B, Schmidt S, Metallo CM, Dranka BP, Schwartz B, DeBerardinis RJ. Reductive carboxylation supports redox homeostasis during anchorage-independent growth. Nature 2016; 532: 255-258.
5) McGarry T, Biniecka M, Veale DJ, Fearon U. Hypoxia, oxidative stress and inflammation. Free Radic Biol Med 2018; 125: 15-24.
6) Fuhrmann DC, Brune B. Mitochondrial composition and function under the control of hypoxia. Redox Biol 2017; 12: 208-215.
7) Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. Science 2011; 333: 1109-1112.
8) Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, Sack MN, Kastner DL, Siegel RM. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). J Exp Med 2011; 208: 519-533.
9) Shearer MJ, Newman P. Metabolism and cell biology of vitamin K. Thromb Haemost 2008; 100: 530-547.
10) Suttie JW. Synthesis of vitamin K-dependent proteins. FASEB J 1993; 7: 445-452.
11) Abdel-Rahman MS, Alkady EA, Ahmed S. Menaquinone-7 as a novel pharmacological therapy in the treatment of rheumatoid arthritis: a clinical study. Eur J Pharmacol 2015; 761: 273-278.
12) Sato T, Schurgers LJ, Uenishi K. Comparison of menaquinone-4 and menaquinone-7 bioavailability in healthy women. Nutr J 2012; 11: 93.
13) Zeidan YH, Hannun YA. Translational aspects of sphingolipid metabolism. Trends Mol Med 2007; 13: 327-336.
14) Tsang CK, Kamei Y. Novel effect of vitamin K(1) (phylloquinone) and vitamin K(2) (menaquinone) on promoting nerve growth factor-mediated neurite outgrowth from PC12D cells. Neurosci Lett 2002; 323: 9-12.
15) Nakajima M, Furukawa S, Hayashi K, Yamada A, Kawashima T, Hayashi Y. Age-dependent survival-promoting activity of vitamin K on cultured CNS neurons. Brain Res Dev Brain Res 1993; 73: 17-23.
16) Li J, Lin JC, Wang H, Peterson JW, Furie BC, Furie B, Booth SL, Volpe JJ, Rosenberg PA. Novel role of vitamin k in preventing oxidative injury to developing oligodendrocytes and neurons. J Neurosci 2003; 23: 5816-5826.
17) Bentley R, Meganathan R. Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiol Rev 1982; 46: 241-280.
18) Vos M, Esposito G, Edirisinghe JN, Vilain S, Haddad DM, Slabbaert JR, Van Meensel S, Schaap O, De Strooper B, Meganathan R, Morais VA, Verstreken P. Vitamin K2 is a mitochondrial electron carrier that rescues pink1 deficiency. Science 2012; 336: 1306-1310.
19) Lasemi R, Kundi M, Moghadam NB, Moshammer H, Hainfellner JA. Vitamin K2 in multiple sclerosis patients. Wien Klin Wochenschr 2018; 130: 307-313.
20) Pan MH, Maresz K, Lee PS, Wu JC, Ho CT, Popko J, Mehta DS, Stohs SJ, Badmaev V. Inhibition of TNF-alpha, IL-1alpha, and IL-1beta by pretreatment of human monocyte-derived macrophages with menaquinone-7 and cell activation with TLR agonists in vitro. J Med Food 2016; 19: 663-669.
21) Sun Y, Yang J, Hu X, Gao X, Li Y, Yu M, Liu S, Lu X, Jin C, Wu S, Cai Y. Lanthanum chloride reduces lactate production in primary culture rat cortical astrocytes and suppresses primary co-culture rat cortical astrocyte-neuron lactate transport. Arch Toxicol 2018; 92: 1407-1419.
22) Figueroa D, Asaduzzaman M, Young F. Real time monitoring and quantification of reactive oxygen species in breast cancer cell line MCF-7 by 2',7'-dichlorofluorescin diacetate (DCFDA) assay. J Pharmacol Toxicol Methods 2018; 94: 26-33.
23) Wang S, Ji LY, Li L, Li JM. Oxidative stress, autophagy and pyroptosis in the neovascularization of oxygeninduced retinopathy in mice. Mol Med Rep 2019; 19: 927-934.
24) Eltzschig HK, Carmeliet P. Hypoxia and inflammation. N Engl J Med 2011; 364: 656-665.

25) Kolmychkova KI, Zhelankin AV, Karagodin VP, Orekhov AN. Mitochondria and inflammation. Patol Fiziol Eksp Ter 2016; 60: 114-121.
26) Ferland G. The discovery of vitamin K and its clinical applications. Ann Nutr Metab 2012; 61: 213-218.
27) Nepal S, Tiruppathi C, Tsukasaki Y, Farahany J, Mittal M, Rehman J, Prockop DJ, Malik AB. STAT6 induces expression of Gas6 in macrophages to clear apoptotic neutrophils and resolve inflammation. Proc Natl Acad Sci U S A 2019; 116: 16513-16518.
28) Kim H, Kim HJ, Lee K, Kim JM, Kim HS, Kim JR, Ha CM, Choi YK, Lee SJ, Kim JY, Harris RA, Jeong D, Lee IK. alpha-Lipoic acid attenuates vascular calcification via reversal of mitochondrial function and restoration of Gas6/Axl/Akt survival pathway. J Cell Mol Med 2012; 16: 273-286.
29) Aschner M, Sonnewald U, Tan KH. Astrocyte modulation of neurotoxic injury. Brain Pathol 2002; 12: 475-481.
30) Kaczor P, Rakus D, Mozrzymas JW. Neuron-astrocyte interaction enhance GABAergic synaptic transmission in a manner dependent on key metabolic enzymes. Front Cell Neurosci 2015; 9: 120.
31) Giffard RG, Swanson RA. Ischemia-induced programmed cell death in astrocytes. Glia 2005; 50: 299-306.
32) Takano T, Oberheim N, Cotrina ML, Nedergaard M. Astrocytes and ischemic injury. Stroke 2009; 40: 8-12.
33) Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, Jewell CM, Johnson ZR, Irvine DJ, Guarente L, Kelleher JK, Vander Heiden MG, Iliopoulos O, Stephanopoulos G. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature 2011; 481: 380-384.
34) Solevag AL, Schmolzer GM, Cheung PY. Novel interventions to reduce oxidative-stress related brain injury in neonatal asphyxia. Free Radic Biol Med 2019; 142: 113-122.
35) Jaafaru MS, Nordin N, Rosli R, Shaari K, Bako HY, Noor NM, Abdull Razis AF. Prospective role of mitochondrial apoptotic pathway in mediating GMG-ITC to reduce cytotoxicity in H2O2-induced oxidative stress in differentiated SH-SY5Y cells. Biomed Pharmacother 2019; 119: 109445.
36) Ferriero DM. Neonatal brain injury. N Engl J Med 2004; 351: 1985-1995.
37) Schurgers LJ, Teunissen KJ, Hamulyak K, Knapen MH, Vik H, Vermeer C. Vitamin K-containing dietary supplements: comparison of synthetic vitamin K1 and natto-derived menaquinone-7. Blood 2007; 109: 3279-3283.
38) Elder SJ, Haytowitz DB, Howe J, Peterson JW, Booth SL. Vitamin k contents of meat, dairy, and fast food in the u.s. Diet. J Agric Food Chem 2006; 54: 463-467.
39) Ferland G. Vitamin K and the nervous system: an overview of its actions. Adv Nutr 2012; 3: 204-212.
40) Ferland G. Vitamin K, an emerging nutrient in brain function. Biofactors 2012; 38: 151-157.
41) Koshihara Y, Hoshi K, Okawara R, Ishibashi H, Yamamoto S. Vitamin K stimulates osteoblastogenesis and inhibits osteoclastogenesis in human bone marrow cell culture. J Endocrinol 2003; 176: 339-348.
42) Urayama S, Kawakami A, Nakashima T, Tsuboi M, Yamasaki S, Hida A, Ichinose Y, Nakamura H, Ejima E, Aoyagi T, Nakamura T, Migita K, Kawabe Y, Eguchi K. Effect of vitamin K2 on osteoblast apoptosis: vitamin K2 inhibits apoptotic cell death of human osteoblasts induced by Fas, proteasome inhibitor, etoposide, and staurosporine. J Lab Clin Med 2000; 136: 181-193.
43) Li J, Wang H, Rosenberg PA. Vitamin K prevents oxidative cell death by inhibiting activation of 12-lipoxygenase in developing oligodendrocytes. J Neurosci Res 2009; 87: 1997-2005.
44) Sakaue M, Mori N, Okazaki M, Kadowaki E, Kaneko T, Hemmi N, Sekiguchi H, Maki T, Ozawa A, Hara S, Arishima K, Yamamoto M. Vitamin K has the potential to protect neurons from methylmercury-induced cell death in vitro. J Neurosci Res 2011; 89: 1052-1058.
45) Saputra WD, Aoyama N, Komai M, Shirakawa H. Menaquinone-4 Suppresses Lipopolysaccharide-Induced Inflammation in MG6 Mouse Microglia-Derived Cells by Inhibiting the NF-kappaB Signaling Pathway. Int J Mol Sci 2019; 20.
46) Ohsaki Y, Shirakawa H, Miura A, Giriwono PE, Sato S, Ohashi A, Iribe M, Goto T, Komai M. Vitamin K suppresses the lipopolysaccharide-induced expression of inflammatory cytokines in cultured macrophage-like cells via the inhibition of the activation of nuclear factor kappaB through the repression of IKKalpha/beta phosphorylation. J Nutr Biochem 2010; 21: 1120-1126.
47) Reddi K, Henderson B, Meghji S, Wilson M, Poole S, Hopper C, Harris M, Hodges SJ. Interleukin 6 production by lipopolysaccharide-stimulated human fibroblasts is potently inhibited by naphthoquinone (vitamin K) compounds. Cytokine 1995; 7: 287-290.
48) Moriya M, Nakatsuji Y, Okuno T, Hamasaki T, Sawada M, Sakoda S. Vitamin K2 ameliorates experimental autoimmune encephalomyelitis in Lewis rats. J Neuroimmunol 2005; 170: 11-20.
49) Ji R, Meng L, Li Q, Lu Q. TAM receptor deficiency affects adult hippocampal neurogenesis. Metab Brain Dis 2015; 30: 633-644.
50) Funakoshi H, Yonemasu T, Nakano T, Matumoto K, Nakamura T. Identification of Gas6, a putative ligand for Sky and Axl receptor tyrosine kinases, as a novel neurotrophic factor for hippocampal neurons. J Neurosci Res 2002; 68: 150-160.
51) Allen MP, Zeng C, Schneider K, Xiong X, Meintzer MK, Bellosta P, Basilico C, Varnum B, Heidenreich KA, Wierman ME. Growth arrest-specific gene 6 (Gas6)/adhesion related kinase (Ark) signaling promotes gonadotropin-releasing hormone neuronal survival via extracellular signal-regulated kinase (ERK) and Akt. Mol Endocrinol 1999; 13: 191-201.
52) Shankar SL, O'Guin K, Cammer M, McMorris FA, Stitt TN, Basch RS, Varnum B, Shafit-Zagardo B. The growth arrest-specific gene product Gas6 promotes the survival of human oligodendrocytes via a phosphatidylinositol 3-kinase-dependent pathway. J Neurosci 2003; 23: 4208-4218.
53) Mattson MP. Pathways towards and away from Alzheimer's disease. Nature 2004; 430: 631-639.

54) Kim KH, Kim EY, Lee SY, Ko JJ, Lee KA. Oocyte cytoplasmic Gas6 and heparan sulfate (HS) are required to establish the open chromatin state in nuclei during remodeling and reprogramming. Cell Physiol Biochem 2018; 45: 37-53.
55) Kim KH, Kim EY, Ko JJ, Lee KA. Gas6 is a reciprocal regulator of mitophagy during mammalian oocyte maturation. Sci Rep 2019; 9: 10343.
56) Binder MD, Cate HS, Prieto AL, Kemper D, Butzkueven H, Gresle MM, Cipriani T, Jokubaitis VG, Carmeliet P, Kilpatrick TJ. Gas6 deficiency increases oligodendrocyte loss and microglial activation in response to cuprizone-induced demyelination. J Neurosci 2008; 28: 5195-5206.
57) Grommes C, Lee CY, Wilkinson BL, Jiang Q, Koenigsknecht-Talboo JL, Varnum B, Landreth GE. Regulation of microglial phagocytosis and inflammatory gene expression by Gas6 acting on the Axl/Mer family of tyrosine kinases. J Neuroimmune Pharmacol 2008; 3: 130-140.