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Neuroprotective Effect of ω-3 Polyunsaturated Fatty Acids on Bilirubin Encephalopathy *In Vitro* and *In Vivo*

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Manuscript Preparation E
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Background: Bilirubin encephalopathy is a serious complication in neonatal jaundice and is associated with high mortality and disability in newborns. The present study aimed to investigate the neuroprotective effects of omega-3 polyunsaturated fatty acids (ω-3 PUFA) on bilirubin encephalopathy *in vitro* and *in vivo*.

Material/Methods: The cytotoxicity of unconjugated bilirubin (UCB) to neurons and neuroprotection of ω-3 PUFA were investigated using MTT assays and apoptosis evaluations. Superoxide dismutase (SOD) and catalase (CAT) enzyme activity were measured to investigate the anti-oxidative effect of ω-3 PUFA. The differences between eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were also compared. The *in vivo* neuroprotective effect of DHA was demonstrated in neonatal rats with bilirubin encephalopathy by bilirubin monitoring, neuron-specific enolase (NSE) monitoring, H&E staining of brain tissue, and apoptosis rate evaluations.

Results: Omega-3 PUFA reduced the rate of apoptosis induced by UCB and increased SOD and CAT enzyme activity for anti-oxidation. DHA did not reduce the bilirubin in the serum of neonatal rats with bilirubin encephalopathy, but did reduce the damage caused by bilirubin with decreased NSE and apoptosis rate as well as improved neuron morphology.

Conclusions: Omega-3 PUFA, particularly DHA, can reduce neurological damage in neonatal rats with bilirubin encephalopathy by increasing anti-apoptosis and anti-oxidation effects against UCB, providing a theoretical basis for the clinical treatment of bilirubin encephalopathy in newborns.

MeSH Keywords: **Dehydroepiandrosterone Sulfate • Fatty Acids, Omega-3 • Kernicterus**

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Background

Neonatal jaundice is a common condition in newborns, occurring as a result of high serum levels of unconjugated bilirubin (UCB) [1]. Under certain circumstances, high levels of UCB cause serious adverse effects, including central nervous system neurotoxicity, also called bilirubin encephalopathy [2]. According to previous reports, UCB is a "double-edged sword" in brain tissues. Lower concentrations of UCB may be neuroprotective either by scavenging nitric oxide (NO) and preventing cytotoxicity, or by facilitating release of NO and activating NO-related protective signals under conditions of NO excessive release or deficiency, respectively. In addition, a small number of UCBs also scavenge cytotoxic radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS), based on both the conjugate double bonds and reactive hydrogen atom that UCBs can donate [3–5]. Excessive UCB levels have neurotoxic effects though several potential mechanisms: (1) UCB can disrupt the cell and mitochondria membranes, leading to phagocytic recognition and cell death; (2) UCB can induce the loss of myelin basic protein, resulting in lack of myelin sheath formation and increased reactivity of astroglia and microglia; (3) UCB-copper (Cu) system and the resulted ROS are responsible for oxidative DNA damage; (4) UCB stimulates the pro-inflammatory cytokines release; (5) UCB inhibits the nerve growth factor- and brain-derived neurotrophic factor-induced activation of pro-survival signals; and (6) UCB can activate caspase-3 and promote apoptosis [6]. Although the mechanism by which neurotoxicity is induced by UCB is not well understood, the oxidative stress and apoptosis induced by UCB may contribute to considerable neurological damage [7]. The destructive process is gradually caused by the gathering, bonding, and deposition of UCB on the neurons. The oxidative stress induced by the deposited UCB triggers the swelling and apoptosis of neurons, resulting in irreversible neural damage [8]. Given the serious consequences of bilirubin encephalopathy in newborns, more research is needed to better understand this condition.

Fatty acids are an important biological compound that play an essential role in many physiological functions [9]. Omega-3 polyunsaturated fatty acids (ω-3 PUFA) are long-chain fatty acids with more than 1 carbon–carbon double bond, which contains α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). EPA and DHA are considered essential fatty acids [10].

The brain is an abundant source of ω-3 PUFA (particularly DHA) [11], which play a vital role in the maintenance of regular cerebral structure and function, including promoting growth in infancy and delaying aging. Research also shows PUFA may be protective against disease-induced neurodegeneration and apoptosis [12]. Although the neuroprotective mechanism of PUFA needs more study, it is understood that the anti-inflammatory and anti-oxidative effects play a crucial role [13]. Research demonstrated that DHA reduces the activity of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) induced by UCB in the hyperbilirubinemia of newborns *in vitro* [14]; however, only limited *in vivo* research on the neuroprotective effect of ω-3 PUFA exists.

In the present study, the neuroprotective effect of ω-3 PUFA against UCB *in vitro* and *in vivo* was investigated to provide a theoretical basis for the clinical treatment of bilirubin encephalopathy in newborns.

Material and Methods

Cell culture

All animal experiments were approved by the Animal Care and Use Committee of Shandong Provincial Hospital Affiliated with Shandong University. Primary cortical neurons were acquired from neonatal SD rats (<24 h) and cultured according to the methods described in previous reports. The brain was extracted from neonatal rats and fragmented in D-hanks solution (Gibco, USA). After stripping of meninges, blood vessels, and subcortical tissues, the cortex was mechanically minced into pieces. Subsequently, these pieces were treated with trypsin (0.25%, Hyclone, USA) at 37°C for 30 min. The isolated cells were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% FBS (Gibco, USA) and 1% penicillin-streptomycin and seeded into 6-well plates pre-coated with poly-D-lysine (PDL, Sigma, USA). Each well contained approximately 5×10^5 cells. After 1 h, the DMEM medium was used instead of neurobasal medium. The cells were incubated in a 5% CO_2 atmosphere at 37°C.

Cell viability evaluations

Primary neurons were seeded in 96-well plates pre-coated with PDL (1×10^4 cells/well). After 24 h, the cells were treated with UCB (concentrations ranging from 0.1 to 500 μM) for 36 h. Primary neurons were also treated with DHA or EPA (concentrations ranging from 0.1 to 100 μM) for 36 h. Cells were then treated with fresh medium containing MTT (5 mg/mL) and incubated for an additional 4 h in 5% CO_2 at 37°C. The medium was then replaced by 120 μL DMSO. The absorbance values at 490 nm of each well were measured using a microplate reader. Cells were treated with IC_{50} values of UCB after 6 h before treatment with DHA or EPA to examine the potential protective effects.

Apoptosis evaluations

Cells were treated with IC_{50} values of UCB with or without 6-h pretreatment of DHA (10 μM) or EPA (10 μM). After incubation

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for 36 h, cells were washed with PBS and treated with trypsin. The apoptosis of cells was determined through Deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) method using an apoptosis detection kit (Vazyme Biotech, China) in accordance with the manufacturer's instructions.

SOD and CAT enzyme activity evaluations

Cells were treated with IC_{50} values of UCB with or without 6-h treatment with DHA (10 μM) or EPA (10 μM). After incubation for 36 h, cells were washed with PBS and treated with trypsin, and the effects of UCB and DHA or EPA on superoxide dismutase (SOD) and catalase (CAT) enzymes activity was detected. SOD and CAT activity was measured using the SOD assay kit (Sigma-Aldrich, USA) and Catalase assay kit (Sigma-Aldrich, USA), respectively.

Animal model

Sixty 7-day-old neonatal SD rats were randomly divided into 3 groups. We dissolved 20 mg of crystal bilirubin in 1 mL of NaOH (0.5 M), with addition of 9 mL deionized water. The pH of this solution was adjusted to 8.5 using HCl (0.5 M). The model of bilirubin encephalopathy was established through intraperitoneal injection of the above solution (120 mg/kg) in 2 groups of SD rats. An equivalent normal saline (NS) was injected in the same way for the control group. For therapeutic purposes, rats in one of the bilirubin encephalopathy model groups received DHA orally (by gavage, 100 mg/Kg) every day for 3 days, while the rats in the other model group were treated with NS in the same way.

Dynamic monitoring of bilirubin and neuron-specific enolase (NSE)

Blood samples were obtained before and every 24 h after treatment (3 rats every time from every group) and the total bilirubin serum levels were measured using a Bilirubinometer (B-105N, Erma, Tokyo, Japan). The serum NSE levels were detected using an ELISA kit (MSK Bio, China). The mean value of the 3 rats at every point were used for data analysis.

Measurement of bilirubin in the brain tissues of rats

Brain tissue was obtained from rats at 0.5 h after treatment and weighed. After making homogenate, acetone was used to extract the bilirubin, and the supernatant samples were assessed using an automatic biochemical analyzer (Beckman, USA).

Histopathological analysis

On the fourth day after different treatments, the brain tissues of 2 rats in each group were extracted and stained with hematoxylin and eosin (H&E) to evaluate cerebral histological changes.

Apoptosis measurements

Bcl-2 and Bax are important regulatory genes for apoptosis. The expression of Bcl-2 and Bax proteins was detected by the immunohistochemical method according to the operating instructions. Cells showing claybank in the cytoplasm were regarded as positive cells. The apoptosis rate was determined through the TUNEL method.

Statistical analysis

All quantitative data is presented as means ±SEM. Statistical *p* values were calculated by two-way ANOVA analysis and the *t* test, as indicated, with Origin Pro 8.5 software.

Results

ω-3 PUFA protected neurons from damage by UCB

The initial aim of this study was to determine the dosage-dependent cytotoxicity of UCB in neurons. As shown in Figure 1A, a significant promotion of cell proliferation was induced under exposure to a lower concentration of UCB. However, the viability of neurons gradually decreased with increasing concentration of UCB. This suggests the cytotoxicity to neurons. Ten μM of UCB resulted in a mortality rate of about 50% for neurons and this concentration was used in subsequent experiments. In contrast, ω-3 PUFA (EPA and DHA) did not kill neurons, even at a high concentration of 100 μM (Figure 1B). Further, DHA promoted the proliferation of neurons. To investigate the neuroprotection effects of ω-3 PUFA, neurons were pretreated with EPA or DHA before exposure to UCB. As shown in Figure 1C, both EPA and DHA significantly reduced the cytotoxicity of UCB, exhibiting a good biocompatibility of ω-3 PUFA. DHA showed more effective neuroprotection compared to EPA under the same concentration (cell viability of 95.2% *vs.* 65.3%, $p<0.01$). Simultaneous pretreatment with both EPA and DHA did not show a significant difference in cell viability when compared with pretreatment with DHA alone.

It was observed that UCB is toxic to neurons and ω-3 PUFA may play a role in protecting neurons from this toxicity. Further experiments aimed to examine if this was a result of apoptosis and whether ω-3 PUFA had a protective effect against UCB-mediated induction of apoptosis in neurons. Figure 1D shows that UCB can induce increased apoptosis of neurons, which can be reduced with pretreatment of ω-3 PUFA, particularly DHA. The above results suggest that ω-3 PUFA has protective effects against UCB-induced apoptosis in neurons and that DHA has a stronger effect compared to EPA.

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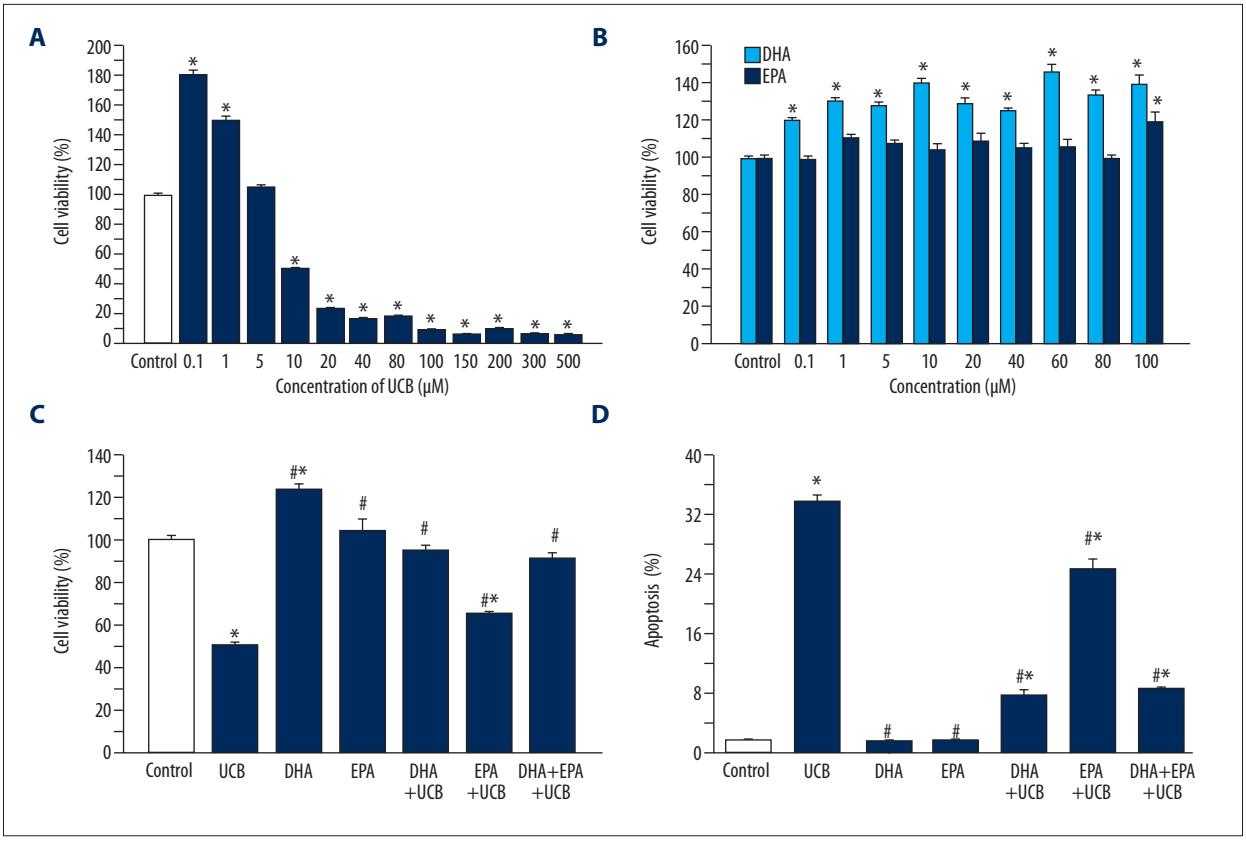

Figure 1. Omega-3 PUFA (DHA and EPA) protected neurons from UCB cytotoxicity. (**A**) Neuronal viability under exposure to UCB with different concentrations (ranging from 0.1 to 500 μM). (**B**) Neuronal viability under exposure to ω-3 PUFA (EPA and DHA) with different concentrations (ranging from 0.1 to 100 μM). (**C**) Neuronal viability under exposure to UCB (10 μM) with different pre-treatments of DHA and EPA (10 μM for each). (**D**) Apoptosis rate of neurons under exposure to UCB (10 μM) with different pre-treatments of DHA and EPA (10 μM for each). (* $p<0.01$ compared to the control group, # $p<0.01$ compared to the UCB group).

ω-3 PUFA showed the antioxidant effect in neuroprotection against UCB

Previous research shows that UCB has oxidant effects, while ω-3 PUFA demonstrates antioxidant properties [7,12]. To further examine this, the effects of UCB and ω-3 PUFA on SOD and CAT enzyme activity in neurons was evaluated. Neurons were exposed to 10 μM UCB with or without pre-incubation of EPA or DHA for 6 h. After 36-h incubation, neurons were collected and lysed. The lysates were used for determining antioxidant enzyme activity. As shown in Figure 2A, UCB decreased SOD activity of neurons compared to the control group. Neurons treated with DHA or EPA alone showed significantly enhanced SOD activity. Similar effects under different treatment conditions were also found in the evaluation of CAT activity (Figure 2B). These results demonstrate that ω-3 PUFA has antioxidant properties, which may play a role in neuroprotection against the oxidative damage caused by UCB. Furthermore, DHA showed a more effective antioxidant ability than EPA. Combination of EPA and DHA for pretreatment of neurons did not significantly enhance the enzyme activity when compared with the DHA pretreatment group. Therefore, the remaining *in vivo* evaluation assay studies focused on the neuroprotective effect of DHA alone.

DHA reduced neurological damage in neonatal rats with bilirubin encephalopathy

The previous experiments validated the neuroprotective characteristics of ω-3 PUFA (especially DHA) from UCB damage via anti-apoptosis effects. Subsequently, DHA was used to investigate neurological protection in the *in vivo* model of bilirubin encephalopathy. The bilirubin encephalopathy model was established using intraperitoneal bilirubin injection. Serum level bilirubin was monitored for 72 h. At 0.5 h after injection, there was a sharp increase in bilirubin serum level (>500 μM; Figure 3A). This was significantly more than in the blank control group (<10 μM). Over time, the serum concentration of bilirubin gradually decreased. The bilirubin encephalopathy rats treated with DHA did not demonstrate a significant reduction

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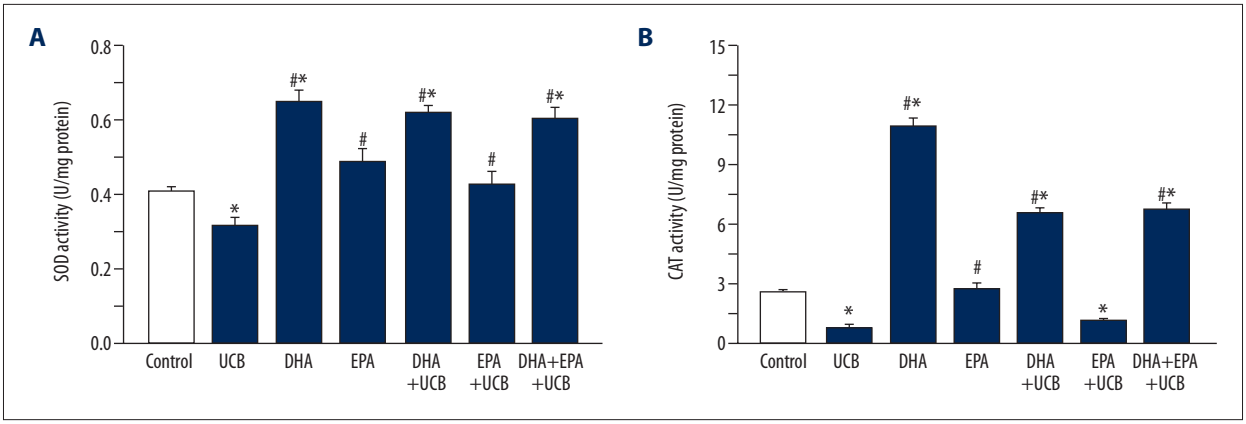

Figure 2. Omega-3 PUFA (DHA and EPA) showed enhanced anti-oxidation to improve oxidative stress induced by the UCB. SOD (**A**) and CAT (**B**) activity under different treatments (UCB: 10 μM; DHA: 10 μM; EPA: 10 μM). (* $p<0.01$ compared to the control group, # $p<0.01$ compared to the UCB group).

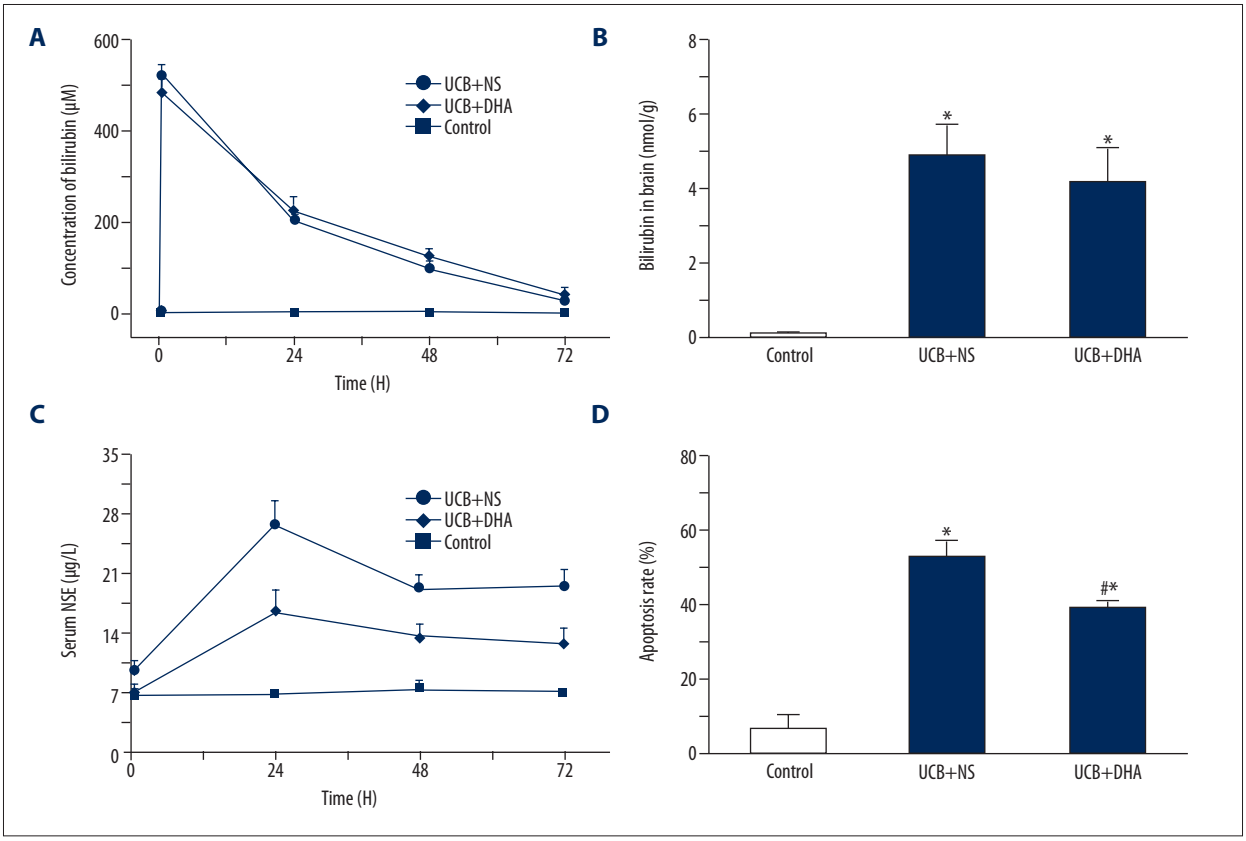

Figure 3. DHA reduced neurological damage in neonatal rats with bilirubin encephalopathy. (**A**) Serum level of bilirubin in the rats at different time points after different treatments (UCB: 120 mg/Kg, intraperitoneal injection; DHA: 100 mg/Kg, by gavage). (**B**) Bilirubin in the brain of mice at 24 h after different administrations (UCB: 120 mg/Kg, intraperitoneal injection; DHA: 100 mg/Kg, by gavage). (**C**) Serum level of NSE in the mice at different time points after different treatments (UCB: 120 mg/Kg, intraperitoneal injection; DHA: 100 mg/Kg, by gavage). (**D**) Apoptosis rate in the cerebral cortex of mice at 72 h after different administrations (UCB: 120 mg/Kg, intraperitoneal injection; DHA: 100 mg/Kg, by gavage). (* $p<0.01$ compared to the control group, # $p<0.01$ compared to the UCB+NS group).

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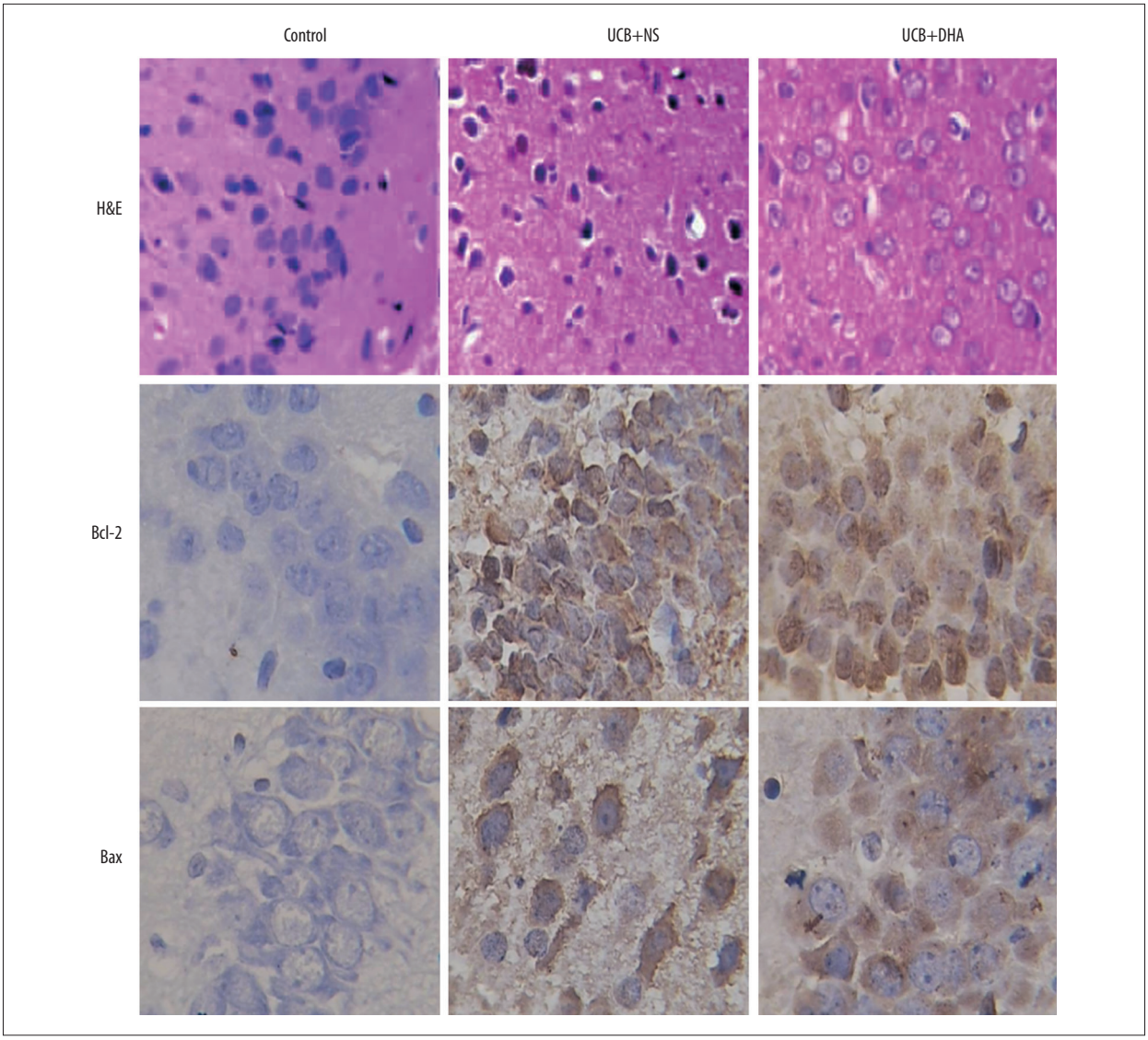

Figure 4. DHA reduced brain tissue damage and apoptosis of neurons in neonatal rats with bilirubin encephalopathy. H&E staining and immuohistochemical staining of Bcl-2 and Bax in the brain tissues of mice with different treatments (UCB: 120 mg/Kg, intraperitoneal injection; DHA: 100 mg/Kg, by gavage).

in bilirubin serum levels compared with the NS-treated model group. The level of bilirubin in the brain tissue was measured at 0.5 h after injection of UCB. As shown in Figure 3B, bilirubin is almost undetectable in the control group mice. However, the brain tissues of UCB-treated mice, with or without administration of DHA, showed significant increases in bilirubin. Enhanced levels bilirubin in the brain tissue confirmed the viability of the bilirubin encephalopathy model described above; however, it demonstrated that DHA did not prevent bilirubin from infiltrating into brain tissue.

NSE is a sensitive indicator of neuron damage. To evaluate of neurological damage, we evaluated the serum levels of NSE in mice. Figure 3C shows that NSE in the serum of UCB and NS-treated mice significantly increased by 72 h and peaked at approximately 24 h after injection. However, NSE in the serum of UCB- and DHA-treated mice was significantly reduced compared with the UCB- and NS-treated groups. These results indicate that bilirubin induces neuron damage, and DHA may play a role in reducing the neurological damage caused by UCB.

To further characterize the neurological damage, the brain tissues of mice at 72 h after injection were extracted and then stained with H&E. As shown in Figure 4, neurons in the control group had a typical arrangement with formal morphology and clear nucleolus. However, neurons in the UCB- and NS-treated groups were reduced in number, had a loose arrangement, and had many vacuoles and dark nuclei. Neurons

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in the DHA-treated group showed improvements in morphology, suggesting there is a neuroprotective effect of DHA at the histological level.

Apoptosis in the damaged brain was investigated using immuohistochemical staining of Bcl-2 and Bax. It can be seen in Figure 4, there was a significant increase in the expressions of Bcl-2 and Bax protein in the UCB- and NS-treated groups. However, the DHA-treated group demonstrated a higher expression of Bcl-2 and a lower expression of Bax compared with the UCB- and NS-treated groups. The apoptosis rate shown in Figure 3D indicates that administration of DHA reduced the apoptosis in the brain induced by UCB. All of the above results suggest that timely treatment with DHA showed a reliable anti-apoptosis and neuroprotective effect in the bilirubin encephalopathy model.

Discussion

Bilirubin encephalopathy is a serious complication of neonatal jaundice [7]. High concentrations of UCB in the blood can easily cross the blood-brain barrier and damage neurons, resulting in neurological disability or even death [15]. Although the mechanism of neurotoxicity induced by UCB is not well understood, previous research indicates that oxidative stress and apoptosis may play a role in damaging neurons [16]. Omega-3 PUFA is an essential nutrient and EPA and DHA play an important role in the protection of cerebral structure and functions [17]. Many reports demonstrate that ω-3 PUFA may have anti-inflammation, antioxidant, and anti-apoptosis characteristics [13]. Although the *in vitro* neuroprotection ability of ω-3 PUFA has been researched at the cellular level [14], few reports have performed *in vivo* evaluations. The present study aimed to investigate the neuroprotective effect of ω-3 PUFA *in vitro* and *in vivo* for bilirubin encephalopathy through the mechanisms of anti-oxidation and anti-apoptosis. This is the first time that ω-3 PUFA has been applied for bilirubin encephalopathy both *in vitro* and *in vivo*.

The human body defends against oxidative stress mainly through 2 mechanisms: enzymes (such as SOD and CAT) and non-enzymatic mechanisms (such as vitamins A, E, and C) [18,19]. Previous reports showed that UCB induces oxidative stress in the cerebral cortex by enhancing formation of protein oxidation, lipid peroxidation, and massive free radicals, leading to DNA damage and cell growth inhibition [20]. In the present study, results show that UCB may inhibit the SOD and CAT enzyme activities in neurons compared with controls. Some reports have suggested that ω-3 PUFA plays a vital role in anti-oxidation by increasing the SOD and CAT activity in different cell lines containing neurons, astrocytes, and ganglion cells [13]. In particular, it was reported that DHA increases SOD and CAT activity in rats with cerebral ischemia [21]. The present study found that ω-3 PUFA (especially DHA) not only significantly increased SOD and CAT activity in neurons, but also reduced the inhibition of antioxidant enzymes activity induced by UCB, which is consistent with previous reports.

In recent studies, neuronal death induced by UCB resulted mainly from apoptosis, likely due to the decrease in mitochondrial permeability and release of cytochrome C [22,23]. Cell apoptosis is a well-organized process of self-destruction, co-regulated by both anti-apoptosis genes and pro-apoptosis genes [24,25]. Among various apoptosis-related proteins, Bcl-2 and Bax are a pair of apoptosis-related proteins in which increased Bcl-2 leads to living cells and Bax results in cell death [26,27]. Recent research showed that Bcl-2/Bax can affect the neurotoxicity of UCB [28,29]. In the present study, the expression of both Bcl-2 and Bax was increased in the cerebral cortex of neonatal rats with bilirubin encephalopathy compared with the control group. However, rats treated with DHA expressed more Bcl-2 and less Bax compared with the bilirubin encephalopathy model group. These results suggest that DHA participates in the up-regulation of anti-apoptosis proteins to counteract the pro-apoptosis effect induced by bilirubin.

Further, DHA demonstrated more effective neuroprotection compared to EPA, and co-administration of both EPA and DHA did not lead to a greater effect. The specific mechanism underlying these differences requires further investigation.

Conclusions

In summary, results demonstrate that ω-3 PUFA, particularly DHA, reduced neurological damage in the neonatal rats with bilirubin encephalopathy through anti-apoptosis and anti-oxidation effects against UCB, providing a theoretical basis for the clinical treatment of bilirubin encephalopathy in newborns.

Conflict of interest

None.

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