Dehydroascorbid Acid Attenuates Ischemic Brain Edema and Neurotoxicity in Cerebral Ischemia: An in vivo Study

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Ischemic stroke results in the diverse pathophysologies including blood brain barrier (BBB) disruption, brain edema, neuronal cell death, and synaptic loss in brain. Vitamin C has known as the potent anti-oxidant having multiple functions in various organs, as well as in brain. Dehydroascorbid acid (DHA) as the oxidized form of ascorbic acid (AA) acts as a cellular protector against oxidative stress and easily enters into the brain compared to AA. To determine the role of DHA on edema formation, neuronal cell death, and synaptic dysfunction following cerebral ischemia, we investigated the infarct size of ischemic brain tissue and measured the expression of aquaporin 1 (AQP-1) as the water channel protein. We also examined the expression of claudin 5 for confirming the BBB breakdown, and the expression of bcl 2 associated X protein (Bax), caspase-3, inducible nitric oxide synthase (iNOS) for checking the effect of DHA on the neurotoxicity. Finally, we examined postsynaptic density protein-95 (PSD-95) expression to confirm the effect of DHA on synaptic dysfunction following ischemic stroke. Based on our findings, we propose that DHA might alleviate the pathogenesis of ischemic brain injury by attenuating edema, neuronal loss, and by improving synaptic connection.

Key words: Dehydroascorbid acid (DHA), Cerebral ischemia, Edema, Blood-brain barrier (BBB), Neurotoxicity, Synaptic dysfunction

INTRODUCTION

Ischemic stroke is the second leading cause of death worldwide accompanied by severe disability [1]. Cerebral ischemia and reperfusion injury leads to damage of brain tissues, inflammation as a result of the blood–brain barrier (BBB) disruption, oxidative damage [2], and apoptosis [3]. Brain tissue is highly vulnerable to oxidative damage because of its high use of oxygen [4] under cerebral ischemia. Cerebral ischemia leads to loss of tight junction proteins in brain endothelium, BBB disruption, and finally brain edema [5]. Brain edema leads to an imbalance in energy demand and influences on the postsynaptic effects of glutamate [6] and interruption of synaptic transmission in the penumbra after stroke [7,8]. Overall, excitotoxicity, inflammation and oxidative stress caused by ischemic stroke plays a crucial role in the pathophysiology of ischemic stroke [9,10]. To reduce the brain damage caused by cerebral ischemia, the solution for...
oxidative damage is the issue of the greatest importance. Vitamin C is the most important antioxidant for metabolic function of the brain [11-13] owing to its low redox potential which is capable of neutralizing diverse pro oxidants [14-17]. Mainly, vitamin C could be found in its form such as ascorbic acid (AA) and dehydroascorbic acid (DHA) (AAs oxidized form) [18,19]. According to earlier studies, lower levels of vitamin C are a risk factor of cerebral stroke [20,21] and actually, decreased vitamin C levels has been demonstrated in patients with ischemic stroke [22]. Recent study demonstrated that the treatment of AA prevented the disruption of BBB and sustained the BBB integrity in the cortex [23]. Neuroprotection by DHA has been demonstrated in several recent studies in both in vitro and in vivo. In in vitro study, DHA has been reported that it inhibits mitochondrial damage and cell death against oxidative injury [24]. Specifically, DHA among vitamin C could crosses the BBB through glucose transporter 1 (GLUT1) [25] and prevents cell death against oxidative damage by increasing glutathione (GSH) levels through glucose transporters [26,27]. In in vivo study, DHA have been reported to have protective effects as antioxidants in experimental neurological disease models such as stroke [19,28-30]. DHA administration attenuates oxidative stress markers and inflammation in hyperglycemic stroke models [31]. However, the study on the role of DHA administration through intra-peritoneal route in ischemic stroke animal model focused on edema formation, neurotoxicity, and synaptic dysfunction has not yet been determined. In present study, we investigated DHA’s beneficial effect after ischemic brain injury in in vivo study. Our results show that DHA is involved in the prevention of brain edema formation, neurotoxicity, and synaptic dysfunction following ischemia injury. Thus, we suggest that DHA might mitigate stroke-induced pathological alterations following cerebral ischemic stroke.

MATERIALS AND METHODS

Animal model
Male Sprague-Dawley (SD) rat (Orient, GyeongGi-Do, Korea; 8 weeks old; 250-260 g) were subjected to transient focal cerebral ischemia by intraluminal middle cerebral artery blockade with a nylon suture, as previously described [32]. After 60 min of middle cerebral artery occlusion (MCAO), blood flow was restored by withdrawing the suture, and regional cerebral blood flow was monitored using a laser Doppler flow meter (Transonic Systems, Inc., Ithaca, NY, USA). All animal procedures and experiments were performed in accordance with the Guide to the Care and Use of Laboratory Animals and were approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

Drug treatments
For each experiment, rats were given anesthesia (chloral hydrate 300 mg/kg/ip). DHA was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA), and dissolved in normal saline (pH 7.5) and administered to rat through intra-peritoneal (i.p) route. Rats were injected with DHA (100 mg/kg/ml) treatment at a time for 10 min just after MCAO occlusion time. Control animals were given an equal volume of saline by the same procedure.

Evaluation of brain edema
For the evaluation of brain edema, mice were sacrificed at reperfusion 24 hr after MCAO injury. Brain slices (2 mm thick) between 22.00 mm and +4.00 mm from Bregma were incubated with 2% 2, 3, 5-triphenyltetraxolium chloride (TTC) (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 10 min in the dark in a drying oven, and later photographed using a Nikon E950 digital camera attached to a dissecting microscope. Infantar volume was determined from digitized images using the Quantity One software package (Bio-Rad, CA, USA). Typically 3 such slices were used for analysis. The area of the cortical and striatal infarct was measured separately in all slices in the ischemic and non-ischemic hemisphere. The ipsilateral and contralateral hemispheres were used to calculate the percentage of brain edema [33].

Cresyl violet staining
At reperfusion 24 hr after MCAO injury, mice were sacrificed and brains were fixed in 3.7% formaldehyde and quickly frozen. Tissues were sectioned coronally at 20 μm thickness and sequentially dipped into xylene 5 min, 100% alcohol 5 min, 95% alcohol 5 min, and 70% alcohol 5 min. Samples were stained with cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 3 min. After the staining, slides were reacted with 70% alcohol 5 min, 95% alcohol 5 min, 100% alcohol 5 min, and xylene 5 min. After these processes, sections were observed under a microscope equipped with a digital camera (Olympus, Tokyo, Japan).

Western blot analysis
At reperfusion 24 hr after MCAO injury, rat were sacrificed and brains were washed rapidly with ice-cold PBS, and collected. Tissues were lysed with ice-cold RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The lysates were centrifuged at 13,200 rpm for 1 hr at 4°C to produce whole-cell extracts. Protein content was quantified using the BCA method (Pierce, Rockford, IL, USA). Protein (20 μg) was separated on a 10% SDS–polyacrylamide (PAGE) gel and transferred onto a polyvinylidene difluoride.
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(313x83)DHA affects the expression of AQP-1 in ischemic brain and may indicate that the DHA treatment reduced brain edema formation after ischemic brain injury.

**RESULTS**

**DHA reduced brain edema formation following cerebral ischemia**

To investigate whether DHA affects vascular permeability in animal brain, we measured brain edema at reperfusion 24 hr after MCAO injury using TTC staining (Fig. 1A). White areas in brain are damaged brain areas due to ischemia (Fig. 1A). Fig. 1B shows the infarct size of brain in both groups (Fig. 1B). The graph shows the percentage of the ipsilateral hemisphere compared with the contralateral hemisphere both in the MCAO and DHA groups (Fig. 1C). The percentage of brain edema in the MCAO group was >12% whereas the percentage of brain edema after DHA treatment was <8% (Fig. 1C). Brain edema (%) was significantly reduced in the DHA group compared with the MCAO group. Our results indicate that the DHA treatment reduced brain edema formation after ischemic brain injury.

**DHA reduced the expression of AQP-1 as the marker of vascular permeability following cerebral ischemia**

We performed immunohistochemistry using AQP-1 antibody at reperfusion 24 hr after MCAO injury to examine whether there were change of markers that affect vascular permeability both in cortex (Fig. 2A) and in striatum (Fig. 2B). We did not observe AQP-1 immunoreactivity in the cortex of the normal control (NC) group (Fig. 2A). However, AQP-1 positive cells were strongly expressed in the cortex in reperfusion 24hr after MCAO injury group (experimental control (EC) group) (Fig. 2A). In addition, DHA treated MCAO rat brain did not exhibit strongly the expression of AQP-1 compared to 24hr MCAO group (EC group). In striatum, AQP-1 expression showed the same pattern as the cortical AQP-1 in reperfusion 24 hr after MCAO injury (Fig. 2). Fig. 2C was significantly shown the decreased fluorescent intensity of AQP-1 over 3 times in the DHA treatment group compared to EC group (Fig. 2C). These data indicate that DHA affects the expression of AQP-1 in ischemic brain and may

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be involved in vascular permeability and edema after ischemia.

**DHA protects blood brain barrier (BBB) disruption following cerebral ischemia**

We conducted immunohistochemistry using Claudin 5 antibody at reperfusion 24 hr after MCAO injury to confirm whether or not there was change of marker as the component junction protein of blood brain barrier (BBB) both in cortex (Fig. 3A) and in striatum (Fig. 3B). In the NC group, Claudin 5 was considerably expressed both in cortex (Fig. 3A) and in striatum (Fig. 3B). However, Claudin 5 expression was evidently attenuated both in cortex (Fig. 3A) and in striatum (Fig. 3B) at reperfusion 24 hr after MCAO injury group (EC group). In the DHA treatment group, Claudin 5 expression was more increased both in cortex (Fig. 3A) and in striatum (Fig. 3B) compared to EC group. Fig. 3C was shown the significantly increased fluorescent intensity of Claudin 5 over 2 times in the DHA treatment group compared to EC group (Fig. 3C). Based on these results, we suggest that DHA may
preserve the expression of Claudin 5 in ischemic brain and also protect the BBB disruption.

**Morphological alteration assessment using cresyl violet staining by DHA treatment**

Cresyl violet staining was performed at reperfusion 24 hr after MCAO injury to assess the extent of ischemia-induced damage histologically in the striatum and cortex (Fig. 4). In the normal control group (without MCAO injury, without DHA treatment), intact cellular structure was observed in both the cortex and striatum. In the MCAO group (EC group), shrunk small cell bodies were detected, and also damaged tissue was observed in the ischemic cortex and striatum (Fig. 4). In DHA group treatment (DHA treatment and MCAO injury), damaged cells were reduced in number compared with EC group, and we observed healthy round cells in the ischemic cortex and striatum (Fig. 4).

**DHA attenuates the cell damage against neurotoxicity following cerebral ischemia**

We performed immunohistochemical staining using cleaved
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Fig. 5. Immunohistochemical image for confirmation of reduced cleaved caspase-3 expression by DHA treatment. (A) Immunohistochemical images showed that cleaved caspase-3 positive cells (red) were densely expressed in the EC group. In DHA treatment group, cleaved caspase-3 expression was decreased in rat cortex, compared with the EC group. (B) In DHA treatment group, cleaved caspase-3 positive cells were decreased in rat striatum due to DHA treatment. (C) The graph showed the percentage (%) of cleaved caspase-3 positive cells to compare the difference of cleaved caspase-3 fluorescence intensity. Statistical significance with EC group was determined by t-test. (D) The graph of cleaved caspase-3 protein level showed the same pattern with immunohistochemical images. Differences were considered significant at *p<0.05, (ANOVA followed by Bonferroni post hoc multiple comparison test). Scale bar=100 μm. Cleaved caspase-3: red, 4', 6-diamidino-2-phenylindole (DAPI): blue. NC: normal control group, EC: experimental control; reperfusion 24 hr after MCAO injury, DHA: DHA treatment and reperfusion 24 hr after MCAO injury.

caspase-3 antibody at reperfusion 24 hr after MCAO injury to examine whether DHA influences on the cell death both in cortex (Fig. 5A) and in striatum (Fig. 5B). Cleaved caspase-3 immunopositive cells were not observed in the rat cortex of the normal control (NC) group (Fig. 5A). However, cleaved caspase-3 positive cells were strongly expressed in the cortex in reperfusion 24 hr after MCAO injury group (EC group) (Fig. 5A). In addition, DHA treated rat cortex did not exhibit the expression of cleaved caspase-3 strongly compared to 24 hr MCAO group (EC group) (Fig. 5A). In striatum, cleaved caspase-3 expression showed the same pattern of the cortex (Fig. 5B). Fig. 5C was shown that the fluorescent intensity of cleaved caspase-3 was attenuated significantly over 4 times in the DHA treatment group compared to EC group (Fig. 5C). The western blot data (Fig. 5D) also showed the reduction of cleaved caspase-3 expression in the DHA treatment group in spite of the MCAO injury. Following these data, DHA may inhibit the cell death under ischemic injury by attenuating the expression of cleaved caspase-3. Second,
we conducted immunohistochemistry using Bax antibody at reperfusion 24 hr after MCAO injury to examine whether DHA influences on the alteration of marker that affects mitochondrial cell death both in cortex (Fig. 6A) and in striatum (Fig. 6B). We did not observe Bax immunoreactivity in the rat cortex of the normal control (NC) group (Fig. 6A). However, the expression of Bax was increased strongly in the cortex at reperfusion 24 hr after MCAO injury group (EC group) (Fig. 6A). In addition, the expression of Bax were not strongly exhibited in the cortex of DHA treated MCAO group compared to 24 hr MCAO group.
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(EC group) (Fig. 6A). In striatum, Bax expression showed the same pattern as the cortex (Fig. 6B). Fig. 6C was shown that the significantly reduced fluorescent intensity of Bax over 2 times in the DHA treatment group compared to EC group (Fig. 6C). The western blot data (Fig. 6D) also showed the reduction of Bax expression in the DHA treatment group compared to the MCAO injury (Fig. 6D). Considering these data, DHA may influence on the cellular protection against the mitochondrial cell death under ischemic injury by attenuating the expression of Bax. Additionally, we conducted immunohistochemistry using iNOS antibody at reperfusion 24 hr after MCAO injury to confirm whether there was change of marker as the inflammatory mediator in both cortex (Fig. 7A) and striatum (Fig. 7B) or not. In the EC group, iNOS was considerably expressed in both in cortex (Fig. 7A) and striatum (Fig. 7B). However, iNOS expression was not found in both cortex (Fig. 7A) and striatum (Fig. 7B) at NC group. In the

![Fig. 7. Immunochemical image for confirmation of attenuated iNOS expression by DHA treatment.](image)

(A) Immunochemical images showed that iNOS positive cells (red) were considerably expressed in the EC group. The rat cortex in DHA treatment group showed reduced iNOS expression compared with EC group. (B) In DHA treatment group, iNOS positive cells were decreased in rat striatum. (C) The graph showed the percentage (%) of iNOS positive cells to compare the difference of iNOS fluorescence intensity. Differences were considered significant at **p<0.01 (t-test via EC group). Scale bar=100 μm, iNOS: inducible nitric oxide synthase (iNOS); red, 4’, 6-diamidino-2-phenylindole (DAPI): blue. NC: normal control group, EC: experimental control; reperfusion 24 hr after MCAO injury, DHA: DHA treatment and reperfusion 24 hr after MCAO injury.

![Fig. 8. Immunochemical image for confirmation of PSD-95 expression by DHA treatment.](image)

(A) Immunochemical images showed that PSD-95 positive cells (red) were few expressed in the EC group cortex. In DHA treatment group, PSD-95 expression was considerably increased in rat cortex compared to the EC group. (B) In DHA treatment group, PSD-95 positive cells were increased in rat striatum owing to DHA treatment. (C) The graph showed the percentage (%) of PSD-95 positive cells to compare the difference of PSD-95 fluorescence intensity. Differences were considered significant at **p<0.01 (t-test via EC group). Scale bar=100 μm, PSD-95: postsynaptic density protein 95 (PSD-95); red, 4’, 6-diamidino-2-phenylindole (DAPI): blue. NC: normal control group, EC: experimental control; reperfusion 24 hr after MCAO injury, DHA: DHA treatment and reperfusion 24 hr after MCAO injury.
DHA treatment group, iNOS expression was decreased in both cortex (Fig. 7A) and striatum (Fig. 7B) compared to EC group. Fig. 7C was shown that the significantly decreased fluorescent intensity of iNOS over 9 times in the DHA treatment group compared to EC group (Fig. 7C). Based on these results, we suggest that DHA may inhibit the expression of iNOS in ischemic brain and also block the expression of inflammatory mediators.

**DHA prevents the damage of post synaptic plasticity following cerebral ischemia**

We performed immunohistochemistry using PSD-95 antibody at reperfusion 24 hr after MCAO injury to check whether DHA affect the postsynaptic plasticity's damage following cerebral ischemia (Fig. 8). We observed strong PSD-95 immunoreactivity in the cortex of the NC group (Fig. 8A). However, PSD-95 positive cells were substantially decreased in the cortex at reperfusion 24 hr after MCAO injury group (EC group) (Fig. 8A). In addition, it showed that strong expression of PSD-95 was observed in DHA treated rat cortex compared to 24 hr MCAO group (EC group). In striatum region, PSD-95 expression showed the same pattern as the cortex (Fig. 8B). Fig. 8C was significantly shown the increased fluorescent intensity of PSD-95 over 6 times in the DHA treatment group compared to EC group (Fig. 8C). Taken together, we speculate that DHA may improve the synaptic dysfunction in ischemic brain.

**DISCUSSION**

Ischemic stroke causes the blockage of cerebral blood vessels in the regions of brain, which can lead to human disability and death [36]. Subsequently, the blockage of blood vessels following stroke leads to diverse pathophysologies including brain edema, neuronal loss, and cognitive dysfunction [37-40]. Cerebral cortex, hippocampus, and corpus striatum in the brain are the most vulnerable regions against oxidative stress and hypoxic injury induced by cerebral ischemia [41]. Many studies has reported that vitamin C among antioxidants is generally neuroprotective in response to brain ischemic injury [42-45]. Oral administration of AA to animal had demonstrated that it suppresses neuronal damage under cerebral ischemia-reperfusion [46]. Dehydroascorbic acid as AA's oxidized form [15,18,19] has been reported that it has a neuroprotective role [47] and is easily transported to the brain by mediating glucose transporter 1 (GLUT1) located in the endothelial cells of the BBB [48]. However, DHA did not fully be investigated in ischemic stroke animal model in spite of its advantages. We anticipated that DHA as an anti-oxidant may considerably affect on cerebral ischemia animal because it can rapidly pass through the brain than AA [25]. In present study, we investigated the neuroprotective effects of brain by DHA i.p administration in cerebral ischemia rat. First, we obtained the consequence that DHA treatment inhibits the brain edema formation in MCAO rat brain. Edema defined as an abnormal increase in brain water content is frequently observed in cerebral ischemia and also has a critical influence on morbidity and mortality [49]. Several studies reported that cerebral ischemia contributes to damage the integrity and permeability of the BBB [50,51]. Aquaporin (AQP) is the water channel protein that facilitates water transport through cell membranes [52,53]. Specifically AQP-1 is permeable only to water and is considered to participate in brain water homeostasis [54]. In addition, AQP-1 has been reported that it is involved in edema formation and cell death in the hippocampus following brain injury [55]. Following our results, we suggest that DHA may reduce osmotic water permeability following cerebral ischemia by inhibiting the expression of AQP-1. All BBB components have been reported to the association with the regulation of the BBB permeability including tight junctions of endothelial cells, glia cells [56-58]. The BBB is composed of the brain endothelial cells interconnected with transmembrane tight junction proteins such as claudin-5 [59] and regulates paracellular permeability [60,61]. In present study, our results indicated that claudin 5 as a tight junction protein in DHA treated MCAO rat brain was evidently preserved against ischemic injury. According to our results, DHA may protect the BBB integrity by preserving tight junction protein in response to ischemic injury. Cerebral ischemia induces the neurotoxic environment in brain and it could result in the severe neuronal cell damage, so we investigated the cell death marker such as Bax [62,63], caspase-3[64,65], and iNOS [66,67] in order to examine the protective effect of DHA against the neurotoxicity following ischemic stroke. In present study, DHA treatment reduced the expression of Bax and caspase-3 which is the marker of the mitochondrial cell death and iNOS in ischemic injured brain. Nitric oxide (NO) that causes neuronal cell death and exacerbates glutamate toxicity after cerebral ischemia [68] is synthesized by NO synthase such as iNOS [69]. Several studies demonstrated that inhibition of iNOS in cerebral ischemia improves neurological deficits and reduces infarct volume [70,71]. In consideration of Figure 1 result, our finding suggested that DHA attenuates the expression of iNOS and it may be linked to reduced infarct volume and improved cell death against hypoxic injury. Additionally, NO formed by iNOS has been reported the implicated in neurodegeneration [69]. Judging from our result regarding the reduced iNOS expression, we suggest the possibility of DHA regarding the improvement of cognitive function against ischemic...
stroke although we did not check the production of NO and behavior test considering that AA improves the cognitive decline in Alzheimer's disease [72]. As mentioned earlier, several studies demonstrated that DHA prevents cell death against ischemic injury [19,28-30]. However, previous studies have not yet been determined the effect of DHA on recovery of neuronal function in ischemia animal model. Therefore, we tried to examine the effect of DHA on neuronal function by measuring indirectly synaptic dysfunction in present study. In order to observe the effect of DHA on ischemia-induced synaptic connection alteration, we investigated the expression of PSD-95 protein in ischemic brain tissue. PSD-95 protein as a postsynaptic marker [73,74] is a member of the membrane-associated guanylate kinase family of synaptic molecules and is localized at excitatory synapses [75]. Postsynaptic densities (PSD) proteins are involved in regulation of synaptic function and in the transduction of synaptic signals to the postsynaptic cell [76-78]. Especially, PSD-95 has been implicated in the regulation of ion-channel function, synaptic activity, and intracellular signaling and finally cognitive impairment [79-81]. In addition, PSD-95 protein is implicated in promoting synapse stability and makes synaptic contacts more stable in neurons [75]. Recent studies suggested that the PSD-95 protein improves the neurophysiologic phenomenon after ischemic stroke involving MCAO [82,83]. Moreover, some researchers demonstrated that the decrease of PSD-95 protein immunoreactivity in the ischemic brain leads to a deficit of postsynaptic plasticity in the brain [84]. Several studies suggest that PSD-95's reduction is associated with cognitive impairment [85-88]. Based on our results, our findings indicate that DHA induced the increase of PSD-95 protein immunoreactivity in ischemic stroke brain and DHA may improve the ischemic-induced synaptic plasticity dysfunction. In addition, although we did not check the memory function related behavior test such as water maze, we carefully expect that DHA may improve the learning and memory dysfunction following cerebral ischemia by promoting the neuron's synapse stability. Taken together, our findings suggest three points that 1) DHA is involved in the inhibition of AQP-1 expression and the preservation of claudin 5, ultimately resulting in the reduction of edema formation induced by cerebral ischemia, 2) DHA is associated with the decrease of Bax, cleaved caspase-3 and iNOS expression, ultimately resulting in the protection of cell death against neurotoxicity following cerebral ischemia, 3) DHA is linked to the preservation of PSD-95 protein expression, ultimately resulting in the improvement of neuron's synaptic connection in cerebral ischemia. The present study has some limitations fully to prove the beneficial effect of DHA against ischemic injury. However, we suggest that this study is worthy in that it provide the need of the further study of DHA on ischemic stroke. Taken together, we propose that the DHA might be beneficial to alleviate clinical pathologies that occur after ischemic stroke.

CONFLICTS OF INTEREST

The authors declare no conflict of interest regarding the publication of this paper.

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