Vitamin D treatment ameliorates memory function through downregulation of BAX and upregulation of SOD2 mRNA expression in transient global brain ischaemic injury in rats

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ABSTRACT

Introduction: Ischaemic stroke induces oxidative stress with SOD2 downregulation, and BAX upregulation producing apoptosis. Vitamin D is a fat-soluble hormone that has a neuroprotective effect. The aim of this study is to elucidate the role of vitamin D in memory function, oxidative stress and apoptosis in transient global brain schaemic injury (TGBIl) model.

Materials and Methods: TGBIl was performed in male Wistar rats (3 to 5 months, 150 to 300 g) which underwent bilateral common carotid artery occlusion (BCCAO) for 20 minutes, then reperfused for 10 days (BCCAO group, n = 6). Two groups of BCCAO were treated with intraperitoneal injection of calcitriol 0.125 µg/kgBW (VD1 group) and 0.5 µg/kgBW (VD2 group). The spatial memory function was tested using a probe test with Morris water maze (MWM). mRNA expression of BAX and SOD2 were assessed by the RT-PCR method. Meanwhile, immunohistochemical staining was used for identification of SOD2 protein. Statistical analysis is tested using one-way ANOVA followed by post-hoc LSD.

Results: MWM showed a shorter duration in target quadrant of BCCAO group than the SO group, which is associated with BAX upregulation and SOD2 downregulation. The VD-treated groups had longer duration probe test compared to BCCAO. Furthermore, VD-treated groups had a longer duration in probe test with lower mRNA expression of BAX and higher expression of SOD2. However, there was no significant difference in VD1 and VD2. Immunostaining showed a reduced SOD2 signal in pyramidal cell of CA1 area in BCCAO group and ameliorated in VD1 and VD2 groups.

Conclusion: Vitamin D ameliorates memory function and attenuates oxidative stress and apoptosis in the TGBIl model.

KEYWORDS:
Vitamin D, memory function, BAX, SOD2, global cerebral Ischaemic

INTRODUCTION

Stroke is a neurological disease characterised by blockage of blood vessels due to clots that form in the brain and disrupt blood flow, block of arteries and cause the rupture of blood vessels, resulting in the sudden death of brain cells due to lack of oxygen.[1] World Health Organisation (WHO) reported that global heart attack and stroke cerebral Ischaemic is the highest cause of death in the world, more than 6 million stroke cases mortality every year.[2]

Global ischaemic is a condition in which blood flow to all areas of the brain is transiently inhibited, resulting in nerve cell death, known as delayed neuronal cell death. Transient global cerebral ischemia model in rat is induced by bilateral common carotid artery occlusion (BCCAO) that blocked blood flow in carotid arteries using non-traumatic vascular clamps for 20 minutes and then releasing the clamps for reperfusion.[5]

Ischaemic reperfusion injury is a common ischemic stroke, in which blood flow is restored (reperfusion) after an ischemic period. Reperfusion can occur spontaneously, through thrombolytic therapy or mechanically. The main mechanisms of reperfusion injury involved oxidative stress, leukocyte infiltration, mitochondrial mechanisms, platelet activation and aggregation, complement activation and disruption of the blood brain barrier (BBB),[6] that will disrupt spatial learning and memory processes.[8]

The cornu ammonis region 1 (CA1) is a pyramidal cell in the hippocampus that is more susceptible to damage and delayed neurological death than the CA3 region when oxygen is deprived. Disruption of CA1 neurons contributes to memory deficits in patients with hippocampal injury.[9] Brain ischemia involves multiple mechanisms, such as excitotoxicity, mitochondrial dysfunction, oxidative stress, inflammation, autophagy and damage to the BBB.[10]

Oxidative stress and neuronal apoptosis are important factors in the pathological process of ischemic reperfusion injury in cerebral ischaemic stroke followed by reperfusion. Oxidative stress refers to a comparative surplus of reactive

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oxygen species (ROS) that is caused by an imbalance between oxidants and antioxidants.[11] Brain tissue is sensitive to oxidative stress because it contains low levels of endogenous antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT), which act as cellular defences against ROS. Oxidative stress is involved in the initiation of apoptosis, furthermore a balance between the anti-apoptotic B cell lymphoma protein (Bcl-2) and the pro-apoptotic regulatory protein BAX regulates apoptosis. BAX mediates caspase-9 activation which upregulates in human brain tissue after ischemia. In ischemic stroke animal, caspase-9 leads to the activation of caspase-3 as the main mediator of apoptosis.[11]

Vitamin D is commonly associated with calcium and bone metabolism, but recently it was found that it is an important component in the development of nerve dysfunction.[12] Vitamin D is an antioxidant in an active form called (calcitriol or 1,25-dihydroxy vitamin D3) and has a neuroprotective effect.[13] Vitamin D can maintain a balance between free radicals and antioxidants by increasing intracellular antioxidant concentrations and eliminating excess free radicals thereby reducing oxidative stress.[14] Vitamin D can suppress BAX activity in the hippocampus, as well as caspase-3 activity through the intrinsic pathway of apoptosis where calcitriol provides a neuroprotective function mouse model of global cerebral ischaemic (GCI).[15] Vitamin D has a neuroprotective effect on hippocampal apoptosis induced by pentylenetetrazole and kainic acid in rats.[16]

Administration of vitamin D increases the expression of ET-1, eNOS and triggers the repair of vascular remodelling in the renal fibrosis model mouse.[17] Stroke patients with low vitamin D levels have a wider infarct volume and worse functional effects.[18] In previous study, it was found that from 30 stroke patients, 65% had decreased vitamin D and 35% had normal vitamin D.[20] Existing studies have not provided an overview of the effect of vitamin D on ischemia conditions in terms of changes to memory, oxidative stress and apoptosis. Therefore, this study was conducted to determine the effect of vitamin D in ischaemic conditions on spatial memory, the apoptotic marker BAX and the antioxidant enzyme SOD2.

MATERIALS AND METHODS

Animal Handling and Transient Global Brain ischaemic Injury Model

The study used 24 male Wistar rats (Rattus norvegicus) aged 2 to 3 months with 150 to 300 g body weight obtained from the University of Muhammadiyah Yogyakarta. This research was approved by the Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Gadjah Mada University on November 24, 2022 with number KE/FK/1486/EC/2022. Rats were randomly divided into four groups (n = 6): SO (Sham operation), BCCAO (transient global cerebral ischemia without vitamin D), VD1 (transient global cerebral ischemia + 0.125 µg/kg/day ip vitamin D injection) and VD2 (transient global cerebral ischemia + 0.5 µg/kg/day ip vitamin D injection).

BCCAO was performed to induce transient brain ischemia. The protocol was based on previous studies.[5,18,19-23] Briefly, the rats were anesthetised using 100 mg/kgBW of ketamine anaesthesia (PT Guardian Pharmatama, Jakarta, Indonesia). During anaesthesia, the anterior midline of the necks of the rats was opened and the common carotid arteries were exposed and clamped using non-traumatic vascular clamps (Dieffenbach, World Precision Instruments, USA). The clamps were left obstructing the blood flow of both common carotid arteries for 20 minutes. Sham operations were carried out on the rats of the SO group. After the surgery, the rats were kept in the recovery phase for 2 days before being tested for spatial memory. The rats were sacrificed on day 10.

Vitamin D Administration

This study used calcitriol (1,25-dihydroxy vitamin D) as active vitamin D in a crystalline solid preparation dissolved in 0.2% ethanol to obtain a concentration of 1 mg/ml. Vitamin D was given at different doses, namely 0.125 µg/kg BW in group 3 and 0.5 µg/kg BW in group 4 by intraperitoneal injection once per day until the rats were determined.

Morris Water Maze Test Probes

Rats were tested for memory with a probe test in a Morris water maze (MWM) for 5 days. From the first day until the fourth day, the rats were allowed to practice finding a foothold (platform) in a fixed position in the MWM pool four times a day then dried and returned to the cage. On the fifth day, the rats were subjected to a probe test, and allowed to swim to find a foothold for 2 minutes, without foothold. As long as the rats are looking for a foothold, the camera is provided to records data on the rat's long track for searching the target quadrant/quadrant 4 (Q4).

Animal Termination and Hippocampus Harvesting

The rats were terminated at 10th day after BCCAO procedure. The rats were anesthetised with ketamine HCl 80 to 100 mg/kgBW by intramuscular injection. After being deeply anesthetised, rats were positioned supine on the operating table, then the abdominal wall was incised from the median line to the right and left lateral sides to open the abdominal and thoracic cavities. The organ perfusion was carried out by flowing 0.9% NaCl solution into the left ventricle. The perfusion process has waited until abdominal visceral organs turned pale, and then the rats were decapitated. The cerebrum is separated from the cranium by cutting the sagittal suture so that the two hemispheres are separated. The dextra hippocampus was separated from the dextra cerebral hemispheres then it was put into RNA later and stored in the refrigerator at -20°C. The left cerebral hemispheres were soaked in 10% formalin in PBS for 24 hours and continued with making paraffin blocks.

Immunohistochemistry SOD2

Four paraffin blocks containing hippocampal tissues were deparaffinised, heated in citrate buffer solution for 20 minutes, and incubated in 3% H2O2 in methanol for 15 minutes. Blocking non-specific antigen are performed using background sniper from the Starr Trek HRP Universal Detection Kit (Biocare Medical®, USA, Cat. #STUHRP700H). Afterwards, the sections were incubated in primary anti-

SOD2 antibody (Bioss, Cat. #bs-1080R, 1:100), overnight at 4°C. On the following day, the sections were incubated in appropriate secondary antibodies for an hour and incubated with avidin-HRP from the Starr Trek HRP Universal Detection Kit (Biocare Medical®, USA, Cat. #STUHRP700H) for 30 minutes. Hippocampal pyramidal cell images are viewed under a digital camera (Optilab, Miconos, Indonesia) connected to a light microscope (Olympus, USA) and a computer with 400x magnification. SOD2 positive cells are brown in the cytoplasm.

RNA Extraction and cDNA Synthesis

The right hippocampus previously kept in RNA later® Stabilization Solution (Thermo Fisher Scientific, USA, Cat. #AM7021) was cut in half. Hippocampal tissue RNA was extracted using the Smobio KIT. The cDNA synthesis process was carried out according to the protocol of ExcelRT™ Reverse Transcription Kit II (Smobio®, Cat. No. RP1400) using extracted RNA. The cDNA results were stored in a refrigerator at -20oC. 3 µl cDNA was mixed with a PCR mixture consisting of 0.8 µl forward primer, 0.8 µl reverse primer, 12.5 µl GoTaq™ Green Master Mix, and 7.9 µl nuclease-free water. The following primers were used (Table I).

RT-PCR machine configuration following initial denaturation 95°C for 2 minutes, denaturation 95°C for 10 seconds, annealing 57.60°C for 1 minute, elongation 72°C for 1-minute, last extension 72°C for 10 minutes, and hold 4°C for 40 cycles. The PCR products were analysed by electrophoresis on 2% agarose gel (Agarose S; Nippon Gene, Tokyo, Japan). The electrophoretic results were photographed by transillumination with ultraviolet light using the Geldoc Syngene Gbox Chemi XRQ Series, then densitometry analysis was measured with ImageJ®.

Analysis of Results and Statistical Tests

Statistical analysis in this study used IBM SPSS Statistics 22 software. The data normality test used the Shapiro-Wilk test for a sample size of less than 50. One-way ANOVA followed by LSD post-hoc multiple comparison analysis was used to examine the mean differences between groups - research subject groups. The mean difference is said to be statistically significant if the p-value < 0.05.

RESULTS

Vitamin D Improved Spatial Memory Function Based on MWM Assessment of Length of Time and Trajectory in the Probe Test

The spatial memory of rats after transient brain ischaemic was evaluated using the MWM procedure consisting of escape acquisition, memory retention and visible platform tests. Probe test analysis revealed that the BCCAO group had a shorter average time in the target quadrant than the SO group. The VD1 and VD2 groups had an average longer time in the target quadrant than the BCCAO group (Figure 1A). One-way ANOVA test revealed that the BCCAO group was significantly different from the SO group ($p = 0.028$), which indicated the memory function in the BCCAO group was worse than the control group. The VD1 group ($p = 0.004$) and the VD2 group ($p = 0.017$) had higher time results and significantly different compared to the time in the BCCAO group. Meanwhile, there was no statistical difference between the two groups given vitamin D.

In the trajectory image, the SO group focused more on the target quadrant, in contrast, in the BCCAO group, the rats circle more around other quadrants than the target quadrant and do not appear to have a search focus on the target quadrant but almost all quadrants are surrounded by rats. In the VD1 and VD2 groups, the rats circled the target quadrant more, and the rats seemed to have a more focused search on the target quadrant (Figure 1B).

Vitamin D Downregulated Expression of BAX and Upregulated Expression of mRNA SOD2 and SOD2 Immunohistochemical Staining

Examination of both mRNA expression of BAX and SOD2 and SOD2 immunohistochemical was assess the effect of vitamin D on apoptotic regulatory markers and antioxidant enzymes. RT-PCR analysis revealed that there is a significant difference between all the groups. The LSD post hoc test showed the expression of BAX mRNA in the BCCAO group ($p = 0.000$) higher than the SO group. Meanwhile, the expression of BAX mRNA in the VD1 ($p = 0.029$) and VD2 group ($p = 0.007$) was lower than in the BCCAO group (Figure 2B). In addition, the LSD post hoc test showed that SOD2 mRNA expression in the BCCAO group ($p = 0.008$) was lower than in the SO group, while SOD2 mRNA expression in the VD1 group ($p = 0.011$) and VD2 ($p = 0.016$) was higher than the BCCAO group (Figure 2C).

DISCUSSION

The main finding of this study is transient brain ischemia using the BCCAO model significantly reduces the memory function that affects the spatial learning ability of rats. This corresponds to a decrease in BAX mRNA expression and an increase in SOD2 mRNA expression.

The BCCAO surgical procedure successfully induces temporary brain ischemia, which causes a decrease in spatial memory function. This finding is similar to several studies that showed memory deficits on the MWM test and damage to the CA1 area of the hippocampus due to BCCAO

Table I: List of primers used in this study

Gen	Primary	No. catalogue
SOD2	F: ATGTTGTGTCGGGCGGCGTGCAGC	IDT - 97228330; 200806011
	R: GCGCCTCCGTGGTACTTCTCTCCGGTG	IDT - 97228329; 200806012
BAX	F: GTGAGCGGCTGCTTGTCT	IDT - 97228333; 180583538
	R: GGTCCCGAAGTAGGAGAGG	IDT - 97228366; 180583539
GAPDH	F: GTTACCAGGGCTGCCTTCTC	IDT - 98517206; 205392190
	R: TCCCGTTGATGACCAGCTTC	IDT - 98517207; 205392191

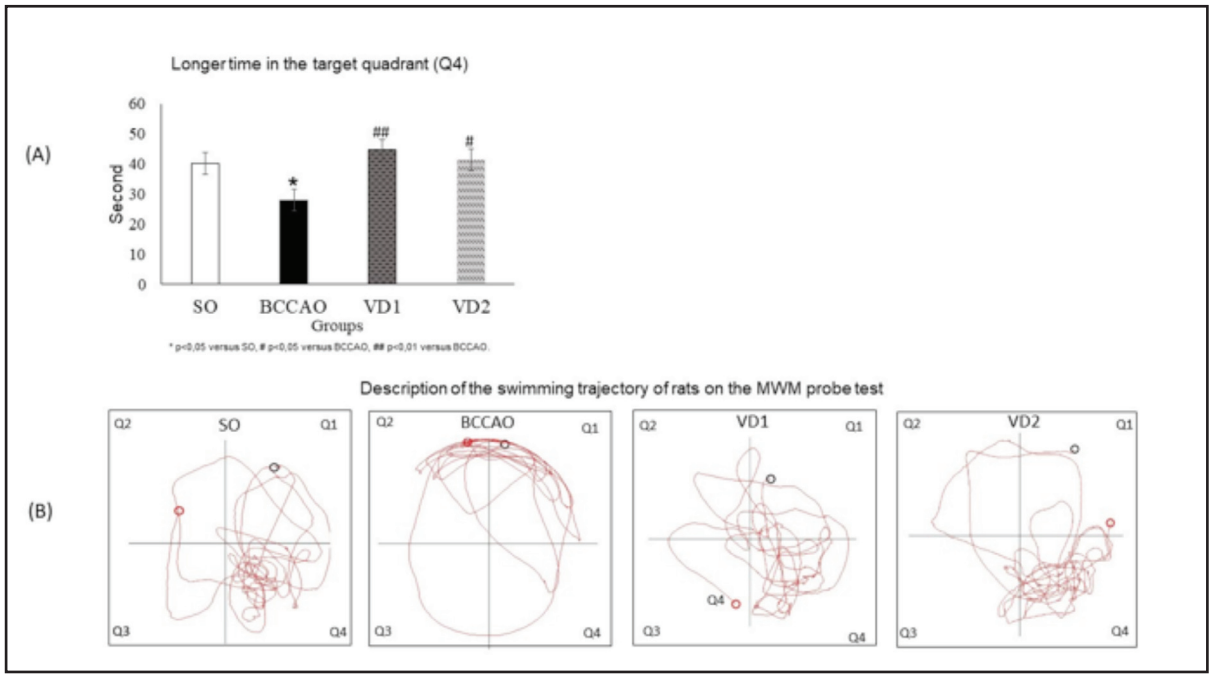

Fig. 1: Graphical representation of the probe test and the probe test path. (A) Graph of the length of time rats spent in the target quadrant (Q4) and (B) Overview of mouse probe test pathways (SO, BCCAO, VD1, VD2)
Notes: (A) * p < 0,05 vs SO, # p < 0.05 vs BCCAO, ## p < 0.01 vs BCCAO. (Analysed by one-way ANOVA, post-hoc LSD test); (B) Target quadrant: quadrant 4 (Q4);O: swimming starting point; O: finishing point of swimming

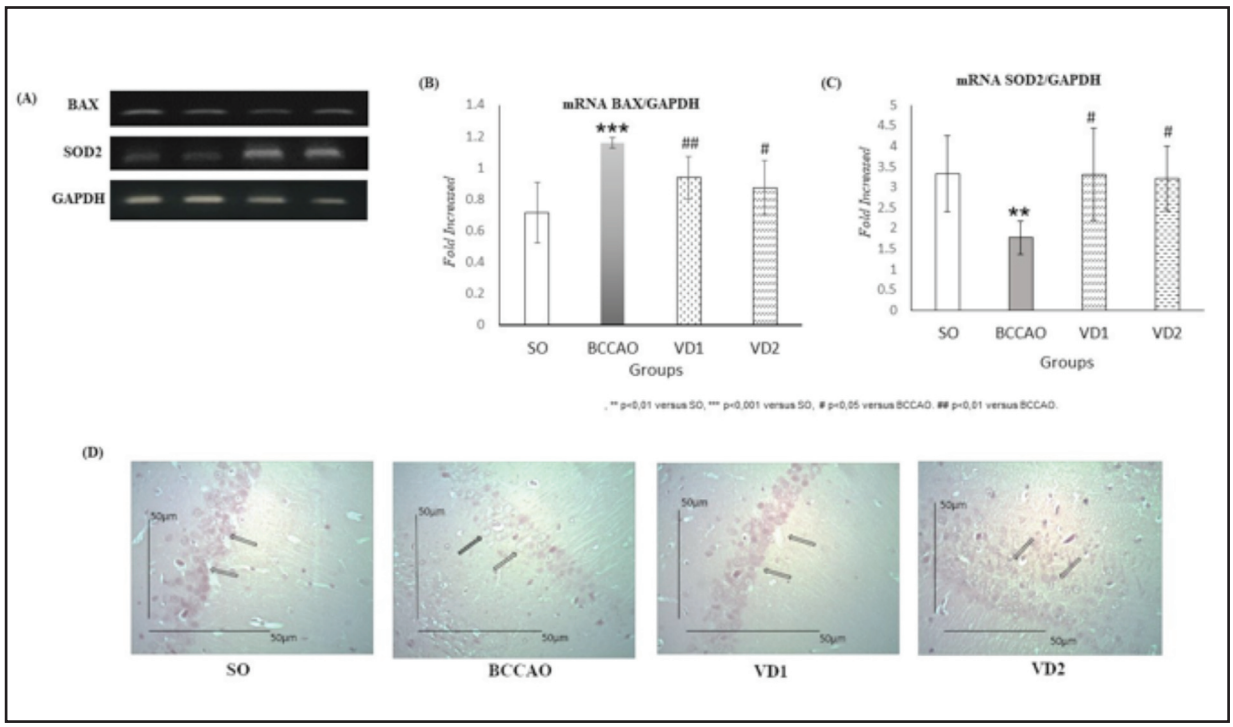

Fig. 2: (A) Representative images of the electrophoresis results of RT-PCR products. (B) Comparison of mean ± SD of BAX/GAPDH mRNA expression. (C) SOD2/GAPDH mRNA expression between the 4 groups of experimental animals. (D) Representative immunohistochemical staining images of the SOD2 enzyme in pyramidal cells of the hippocampal CA1 region. Arrows indicate the expression of the antioxidant enzyme SOD2 as brown spots in the pyramidal cells of the CA1 region of the hippocampus.
Notes: (B, C) ***p < 0.001 vs SO; # p < 0.05 vs BCCAO; ## p < 0.01 vs BCCAO; (Analysed by one-way ANOVA, post-hoc LSD test); (D): Magnification: 400x. ×; Scale bars 50 µm

procedure.[5,23,26-30] It was observed that the learning performance of rat from the ischemic group (BCCAO) tended to be worse than the SO group on the MWM probe test (Figure 1). The decreased spatial memory in rats likely stems from damage to the CA1 region of the hippocampus which is more severely affected by transient brain ischaemic. Therefore, several studies have reported that the CA1 region is more susceptible than the CA2-CA3 region in response to ischaemic.[5,31-33] Transient global cerebral ischemia induced by the BCCAO technique causes a detrimental effect on the brain called reperfusion injury.[6] Reperfusion ischemia injury results in increased production of free radicals which will induce apoptosis of neuron cells, especially in vulnerable areas, namely the hippocampus.[4,5]

The results of the probe test show that the VD1 and VD2 groups had a better memory retention function in the MWM probe test compared to the BCCAO group. Based on the trajectory pattern of the rats in the memory retention test, it was found that the SO rats swam more directionally, heading straight for the target quadrant and swimming more in the target quadrant locations. BCCAO group swam thematically, and the rats have difficulty finding the target quadrant's location. Rats in the VD1 and VD2 groups swam more directionally, finding the target quadrant and swam more in the target quadrant locations (Figure 1A). Based on the pattern of the swimming trajectories of the rats in this study, the SO group and the VD-treated groups seemed to be swimming using the hippocampus-dependent allocentric swimming strategy. In contrast, the BCCAO group appeared to use the hippocampus-independent egocentric swimming strategy (Figure 1B).[34] This suggests that global cerebral ischemia induction performed by the BCCAO technique disrupts spatial memory function according to previous studies.[5,8,35,36]

Different doses are given to determine the effect of the dose. At both doses, there is no significant difference in improving spatial memory function after transient global cerebral ischemia. This aligns with other studies which compared 0.125 µg/kgBW, 0.25 µg/kgBW and 0.5 µg/kgBW doses of vitamin D on fibroblast expansion, inflammation and apoptosis of kidney epithelial cells in UUO models and found that there was no significant difference between the three dose groups.[17] The group that was given vitamin D had a spatial memory function that comparable to the control group which did not undergo ischemia (SO). This demonstrated that vitamin D could restore spatial memory function in global cerebral ischemia until comparable to normal conditions.[37]

A low diet of vitamin D impairs spatial memory function in adult rat. Improvement of spatial memory function in the transient global cerebral ischemia VD-treated showed that vitamin D can induce protection against oxidative stress by upregulating antioxidant proteins.[25,38]

In this study, BAX mRNA expression in the BCCAO group was higher than in the SO group. While BAX mRNA expression in VD1 and VD2 was significantly lower than BCCAO group (Figure 2B). During cerebral ischemia, an imbalance of ionic gradients occurs, depolarising neurons and causing neurotransmitter release, which leads to increased accumulation of glutamate in the extracellular space. Glutamate activates ionotropic glutamate receptors (N-methyl-D-aspartate receptors/NMDAR), which act as excitotoxic channels and allow entry of glutamate ions. Bond of glutamate to NMDAR affects ischaemic-induced Ca^{2+}, which impacts the increased intracellular Ca^{2+} and accumulation of ROS, activating calpains and mediating the cleavage of Bid to tBid, which integrates the different death pathways. At the mitochondrial membrane, tBid interacts with BAX, and BAX forms pores in the mitochondrial outer membrane, releasing Cytc, which executes caspase-dependent cell death. Upon release into the cytosol, the Cytc and procaspase-9 complex form the apoptosome that activates execution caspase, such as caspase-3.[39] Administration of vitamin D protects nerve cells by preventing cytotoxicity and apoptosis, and downregulating L-type voltage-sensitive calcium channels A1C (LVSCC A1C) and upregulating VDR. Vitamin D administration protects against glucocorticoid-induced apoptosis in hippocampal cells, representing vitamin D-mediated neuroprotection.[40]

Calcitriol or 1,25-dihydroxyvitamin D3 is a metabolite of the active form of vitamin D in the kidney.[17] Vitamin D has a mechanism as a neuroprotection that can reduce eruptions and affect complications after a stroke. Another study reported that serum 25(OH)D concentration was inversely related to ischemic infarction volume. In previous studies, vitamin D protects nerves from ischaemic, including postischemic inflammatory response.[3] Vitamin D can suppress BAX activity in the hippocampus, and caspase-3 activity through the intrinsic pathway of apoptosis, where calcitriol provides a neuroprotective function in the mouse model of global cerebral ischemia (GCI).[15] Vitamin D has a neuroprotective effect on hippocampal apoptosis induced by pentylenetetrazole and kainic acid in rat.[16]

The mechanism of vitamin D that can reduce the neurotoxic effects due to oxidative stress can be explained through the following three mechanisms: First, the protective effect of antioxidants against ethanol-induced oxidative stress may stem from the modulation of the expression of survival-enhancing molecules, for example the BCL-2 gene family.[4] Several studies proved that survival-enhancing proteins, such as Bcl-2 and Bcl Xl, play a role in the antioxidant pathway to inhibit apoptosis and oxidation processes, such as lipid peroxidation. Second, vitamin D acts as a membrane antioxidant that protects neurons from damage caused by oxidative stress. Vitamin D accumulates in cell membranes and reduces lipid peroxidation. Several studies suggest that the antioxidant function of vitamin D may be more potent than vitamin E, melatonin and oestrogen. Third, vitamin D has a protective effect by regulating Ca^{2+} homeostasis in brain cells. Vitamin D is known to influence Ca2+ uptake in some inducible cells and to modulate voltage sensitive Ca^{2+}. This demonstrated vitamin D may have a potent neuroprotective effect against glutamate-mediated cytotoxicity.[4,25]

In this study, the expression of SOD2 mRNA in the BCCAO group was lower than the SO group. In contrast, the expression of SOD2 mRNA in the VD1 and VD2 groups was

higher than the BCCAO group (Figure 2C). This study revealed that the localisation of SOD2 expression in the BCCAO group was less than the SO group. SOD2 expression in the VD1 and VD2 groups did not appear to be more than in the BCCAO group. Long-term administration of vitamin D decreases the expression of caveolin-1 (Cav1/L-VGCC subunit) which reduces calcium influx in response to ROS, inflammation and stress.[7] ROS causes translocation of NF-kB to the nucleus and produces proinflammatory cytokines (TNFα, IL-6, IL1β). 1,25(OH)2D3 plays a role in inhibiting NF-kB translocation from the cytoplasm to the nucleus impacting NF-kB expression, reducing iNOS expression and reducing NO production.[7,25]

Previous research stated that vitamin D increases the expression of glutathione peroxidase, which converts ROS H_2O_2 molecules into water. Vitamin D affects the formation of glutathione by the activating the enzyme glucose-6 phosphate dehydrogenase, downregulating NOX to produce ROS, converting O_2 to H_2O_2 and increasing SOD. Vitamin D administration collectively reduces the intracellular burden of ROS.[24] Increased SOD expression protects against brain damage in cerebral ischemia by reducing oxidative injury and modifying redox signals. SOD also reduces mitochondrial dysfunction and subsequent apoptosis after cerebral ischemia. In contrast, NOX, a pro-oxidant enzyme, exacerbates cerebral oxidative stress and contributes to ischemic brain damage.[20]

Vitamin D can maintain the balance between free radicals and antioxidants by increasing intracellular antioxidant concentrations and eliminating excess free radicals thereby reducing oxidative stress.[15] Stroke patients with low vitamin D levels have a more expansive infarct volume and worse functional effects. Another study reported that the serum concentration of 25(OH)D was inversely related to ischemic infarction volume.[3] In previous studies, it was stated that vitamin D protects nerves after ischaemic, inflammation.[3]

CONCLUSSION

Vitamin D has neuroprotective effect on the hippocampus and also ameliorates memory function through downregulation of BAX mRNA expression and upregulation of SOD2 mRNA expression in transient rat models of global cerebral ischemia. Vitamin D could improve antioxidant defence and reduce neuronal apoptosis, thereby preserving memory function.

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