European Review for Medical and Pharmacological Sciences 2022; 26: 2702-2711

Inhibition of A2AR gene methylation alleviates white matter lesions in chronic cerebral hypoperfusion rats

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Abstract. – OBJECTIVE: Chronic cerebral hypoperfusion (CCH) can cause ischemic cerebral white matter lesions (IWML). The aim of this study was to explore the roles of A2A receptors (A2AR) in IWML and the effect of methylation in A2AR gene.

MATERIALS AND METHODS: SD rat model of CCH was constructed by bilateral common carotid artery occlusion (BCCAO) method. The rats were then treated with DNA methyltransferase (DNMT) inhibitor (decitabine), agonist (CGS21680) and A2AR inhibitor (SCH58261). Morris water maze and Kluver-Barrera staining were used to assess spatial learning and reference memory after IWML, respectively. Gene transcription and protein expression were measured by qRT-PCR, Enzyme-linked immunosorbent assay (ELISA) and Western blotting, respectively. The concentration of malondialdehyde (MDA), activity of superoxide dismutase (SOD) and DNMT were detected by assay kit. Methylation of A2AR gene promoter region was detected by bisulfite sequencing PCR (BSP).

RESULTS: We found that the down-regulated expression of A2AR in corpus callosum under CCH was associated with IWML and cognitive impairment. We further showed that A2AR agonist can reduce the IWML under CCH, and A2AR inhibitor can aggravate the IWML under CCH. We also found that the expression level of DNMTs in corpus callosum and the methylation level in the promoter region of A2AR gene were increased under CCH. DNMT inhibitors could protect white matter by inhibiting the methylation of A2AR promoter and rescue the downregulation of A2AR under CCH.

CONCLUSIONS: Our results demonstrate that the downregulation of A2AR mediates IWML in CCH, and A2AR downregulation is related to the increased methylation of A2AR gene promoter. DNMT inhibitors play a protective role in IWML.

Key Words:
A2A receptor, Methylation, Ischemic white matter lesions, Chronic cerebral hypoperfusion.

Introduction

Chronic cerebral hypoperfusion (CCH) can cause ischemic cerebral white matter lesions (IWML), which are shown as hyperintensities on T2 weighted and fluid-attenuated inversion recovery (FLAIR) sequences of MRI. This phenomenon is very common in the aging brain[1], with the prevalence of 92% in 65-75 age group[2], and 58.3% in 40+ age group[3]. Cognitive impairment caused by IWML is one of the main pathological types of vascular dementia, which brings heavy burdens to family and society[4,5]. However, there is still no effective prevention and treatment methods for IWML.

Studies[6] have shown that the white matter lesions caused by CCH involve multiple mechanisms, such as oxidative stress, neuroinflammation, neurotransmitter disorders, mitochondrial dysfunction and lipid metabolism disorders, among which neuroinflammation plays an important role[7] and oxidative stress may be the upstream initiator. Recent studies[8] have shown that adenosine and its receptors, especially A2A receptors (A2AR), are involved in the

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pathological process of IWML. Previous studies[9] showed that macrophage infiltration was observed in the white matter lesions area, and A2AR expression is significantly downregulated. Global A2AR-knockout aggravates chronic hypoperfusion-induced white matter injury by enhancing local inflammatory response[9]. However, the mechanisms of A2AR downregulation under CCH remain unclear. Therefore, this study intends to explore the roles and mechanisms of A2AR in CCH white matter injury, in order to provide theoretical and experimental basis for further research.

Materials and Methods

Ethics and Animals

SD rats (male, 8-week-old, average weight 200 ± 20 g, n = 130) were obtained from Animal Experiment Center of Third Military Medical University. All rats were maintained on a 12 h light/dark schedule, at room temperature (22 ± 2°C) and given food and water *ad libitum* in the Animal Experiment Center of Third Military Medical University. The protocol of animal use was reviewed and approved by the Animal Care and Use Committees of the Third Military Medical University (AMUWEC20210274).

CCH Model

The CCH animal model was constructed by bilateral common carotid artery occlusion (BCCAO) method as following[10]: after anesthesia with 1% sodium pentobarbital (50 mg/kg, i.p.), the animals were kept on a heating pad. Then a mid-neck incision was made 1-1.5 cm above the xiphoid process, and both sides of the common carotid artery were fully dissociated. One side of the common carotid artery was ligated with medical silk thread, and the opposite side of the common carotid artery was ligated with the same method after 30 min, and the wound was finally sutured. The Sham group was subjected to the same surgical procedure without carotid ligation.

The CCH+inhibitor group was treated with SCH58261 (Tocris, Minneapolis, MN, USA) 0.01 mg/kg through intraperitoneal injection, at first 15 min before the surgery, then once a day. The CCH+agonist group was treated with CGS21680 (Tocris, Minneapolis, MN, USA) 0.1 mg/kg through intraperitoneal injection, at first, 15 min before the surgery, and then, once a day. The CCH+DNMT inhibitor group was treated with decitabine (Sigma-Aldrich, St. Louis, MO, USA) 0.4 mg/kg, intraperitoneal injection, at first, 15 min before the surgery, then, once a day. The animals in CCH group and Sham group received equal volume dimethyl sulfoxide (DMSO) through intraperitoneal injection, at first 15 min before the surgery, then once a day.

Morris Water Maze Test

Water maze (diameter 160 cm, height 50 cm) was divided into four quadrants with water injection depth of 30 cm, water temperature (19-22°C), and mildly fixed indirect lighting was provided. The platform (12 cm in diameter) submerged 1-2 cm below the surface of the water and was placed at the center of the third quadrant. Rats were trained to enter the maze from different quadrants to find the platform. After boarding the platform, the recording stopped for 15 s. Rats that could not find the platform within 2 min limit were either placed on the platform or guided to it. The training was performed for 5 days, 4 times a day with 15-20 min intervals. After removing the platform on day 6, the probe experiment was conducted to evaluate the memory of the platform position of the rats[11]. EthoVisionXT 15 software was used for data recording and analysis.

Kluver-Barrera Staining

Rat brain tissue was processed into coronal sections (10 μm). Kluver-Barrera staining (K-B staining) was performed using a myelin sheath dye solution kit (Servicebio, Wuhan, Hubei, China), according to the manufacturer's protocol. Then the stained brain slices were observed using an Olympus microscope.

Malondialdehyde (MDA) and Superoxide Dismutase (SOD) Detection

Rats were anesthetized and perfused with normal saline through heart, then the brain was collected. The brain tissue was homogenized and centrifuged, and the supernatant was collected. The concentration of total proteins was determined by bicinchoninic acid (BCA) method. The concentration of MDA was detected using thiobarbituric acid (TBA) method with lipid peroxidation MDA assay kit (Beyotime, Songjiang, Shanghai, China). The enzyme activity of SOD was detected using WST-8 method with total superoxide dismutase assay kit (Beyotime, Songjiang, Shanghai, China). The experimental protocol

was carried out according to the kit manufacturer's instructions.

Western Blotting

Proteins extracted from cerebral white matter tissues were resolved by Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in block buffer, membranes were incubated with the corresponding primary antibody (rabbit anti-A2AR, Abcam, Cambridge, MA, USA, 1:1000; rabbit anti-DNMT1, CST, Danvers, MA, USA, 1:1000; rabbit anti-DNMT3a, CST, Danvers, MA, USA, 1:1000; mouse anti-DNMT3b, Abcam, Cambridge, MA, USA, 1:1000; mouse anti-GAPDH, Boster, Wuhan, Hubei, China, 1:1000; mouse anti-β-actin, Boster, Wuhan, Hubei, China, 1:1000) at 4°C overnight. Membranes were then incubated with the secondary antibody (HRP conjugated goat anti-rabbit or goat anti-mouse IgG (LiankeBio, Hangzhou, Zhejiang, China, 1:5000) at room temperature for 1 h in the dark. Finally, the band intensity was detected on membranes with chemiluminescence detection system.

Quantitative Real-Time PCR (qRT-PCR)

The tissue of cerebral white matter was homogenized in RNA extraction solution (Servicebio, Wuhan, Hubei, China). Reverse transcription reactions and real-time PCR were performed according to the kit manufacturer's instructions (Takara Biotechnology, Dalian, Liaoning, China). All gene transcription levels were normalized by glyceraldheyde 3-phosphate dehydrogenase (GAPDH), and the relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ formula. The primer sequences used for real-time PCR are available in Table I.

Enzyme-Linked Immunosorbent Assay (ELISA)

Rat A2AR enzyme-linked immunosorbent assay kit (mlbio, Minhang, Shanghai, China) was used to detect A2AR protein expression in rat serum. The detection method was performed according to the manufacturer's protocol.

DNA Methyltransferase Activity Assay

Before assaying DNA methyltransferase (DNMT) activity, nuclear protein was extracted from frozen brain tissue using a nuclear extraction kit (Abcam, Cambridge, MA, USA) following the manufacturer's recommendations. Subsequently, protein concentration of the nuclear extract was measured using Bradford method. Global DNMTs activity of nuclear isolates was assessed using a DNMTs activity assay kit (Abcam, Cambridge, MA, USA), whose experimental protocol follows the manufacturer's instructions.

Bisulfite Sequencing PCR (BSP)

DNA methylation in the promoter region of A2AR gene in Sham group and CCH group was detected by BSP method. Tissue DNA extraction kit (Tiangen Biotechnology, Changping, Beijing, China) was used to extract DNA from brain tissue. The genomic DNA was treated by sulfite with DNA bisulfite conversion kit (Tiangen Biotechnology, Changping, Beijing, China). Then, PCR amplification was performed with PCR kit (Tiangen Biotechnology, Changping, Beijing, China). The primers of A2AR gene promoter region were as follows:

Forward primer: 5'-AGAAAGGTTTTGGTTATTGATGTTT-3', Reverse primer: 5'-ATACCACTACCTCACAATCTTAACTC-3', 206bp.

Finally, PCR products were sequenced to determine whether the CpG site was methylated

Table I. Primer sequences used for quantitative real-time PCR.

Gene	Primer	Sequence	Size
A2AR	F	5'-TCCCCTTCGCTATCACCATC -3'	167bp
	R	5'-CAAGCCATTGTACCGGAGTG -3'	
DNMT1	F	5'-CGGAGACCCAAGGAAGAAGT-3'	187bp
	R	5'-GGTTGAGCTTTGGGATTGCA-3'	
DNMT3a	F	5'-GGTGCTTTTGTGTGGAGTGT-3'	165bp
	R	5'-TGGCGAAGAACATCTGGAGT-3'	
DNMT3b	F	5'-GGACGTGGTAGGAGATGGAG-3'	245bp
	R	5'-ACTTGGTAGCTGGGAACTCC-3'	
GAPDH	F	5'-ACAGCAACAGGGTGGTGGAC-3'	252bp
	R	5'- TTTGAGGGTGCAGCGAACTT-3'	

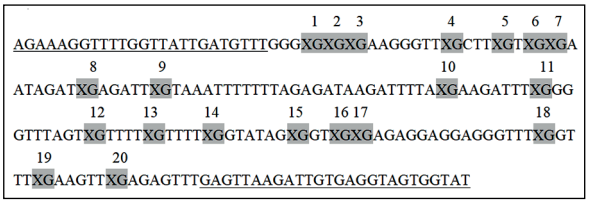

Figure 1. The distribution of 20 CpG sites in the promoter region of A2AR gene.

(Figure 1). According to the sequencing peak diagram (reverse sequencing), the peak height of G (representing the methylated copy number) and the peak height of A (the non-methylated copy number) were calculated, and the methylation percentage of gene promoter was calculated by the following formula: methylation degree (%) = G peak height/(G peak height + A peak height).

Statistical Analysis

All data are expressed as mean±standard error of the mean (SEM) or as percentages. Differences between the two groups were compared using unpaired *t*-test. Differences between multiple groups were compared using one-way or two-way ANOVA. Statistically significant differences between two groups were presented as *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$ and ***, $p<0.001$. All statistical analyses were performed using GraphPad Prism 9.3 (GraphPad Software, San Diego, CA, USA).

Results

White Matter Lesions and Cognitive Impairment Were Associated with A2AR Downregulation in Rats Under CCH

CCH Induced Cognitive Impairment in Rats

Morris water maze was used to assess spatial learning and reference memory in rats. Compared with Sham group, the escape latency time of CCH group was significantly increased at 2 W ($p < 0.01$), 4 W ($p < 0.001$) and 8 W ($p < 0.01$) after surgery (Figure 2 A). Compared with Sham group, the number of CCH group crossing the platform significantly decreased at 4 W ($p < 0.05$) and 8 W ($p < 0.05$) after surgery (Figure 2 B).

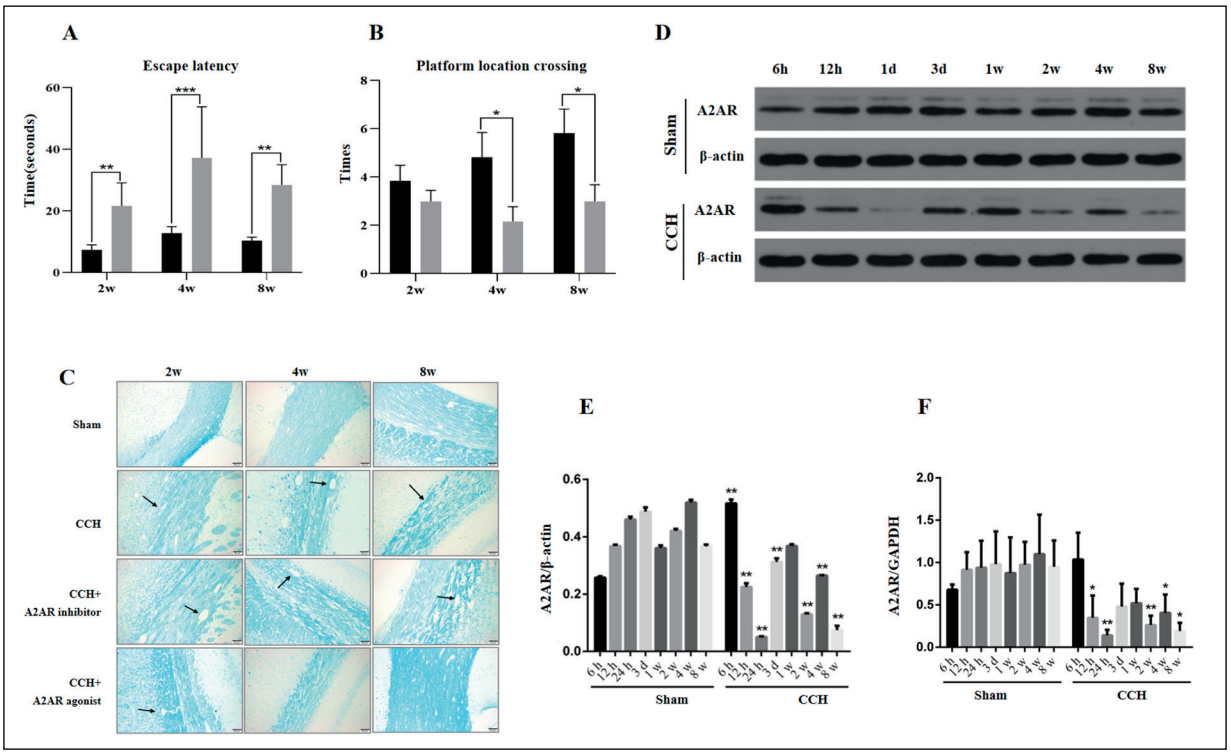

Figure 2. A, The escape latency time of water maze (n = 6). **B,** The number of crossing the platform in water maze (n = 6). **C,** K-B staining of pathological sections in cerebral white matter. **D-E,** Expression of A2AR in corpus callosum was detected by Western blotting (n = 3). **F,** Corpus callosum mRNA level of A2AR was measured by qRT-PCR (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, scale bars represent 50 μm).

CCH Induced White Matter Nerve Fibers Damage

White matter lesions for each animal were evaluated by Kluver-Barrera staining. Compared with Sham group, nerve fibers of CCH group rats became loose, some fibers were disordered, and local cavitation appeared, and the changes were more obvious with the prolongation of chronic cerebral hypoperfusion (Figure 2 C).

CCH led to Decreased Expression of A2AR Gene in Corpus Callosum Region of Rats

The transcription of A2AR gene in corpus callosum for each animal was evaluated by qRT-PCR. Compared with Sham group, the transcription of A2AR gene declined 12 h after surgery in CCH group, and there were significant differences between the two groups at 2 W ($p < 0.01$), 4 W ($p < 0.05$) and 8 W ($p < 0.05$) after surgery (Figure 2 F).

The expression of A2AR protein in corpus callosum was evaluated by Western blotting. Compared with Sham group, the expression of A2AR decreased 12 h after surgery in CCH group, and there were significant differences between the two groups at 2 W ($p < 0.01$), 4 W ($p < 0.01$) and 8 W after surgery ($p < 0.01$) (Figure 2 D-E).

The Upregulation of DNMTs Expression Mediated the Hypermethylation in the Promoter Region of A2AR Gene Under CCH

Oxidative Stress Damage was Induced by CCH

Oxidative stress was measured by MDA and SOD assay. Compared with Sham group, the concentration of MDA increased 12 h after surgery in CCH group, and there were significant differences at 1W ($p < 0.01$), 2 W ($p < 0.01$) and 4 W after surgery ($p < 0.01$) (Figure 3 H). Compared with Sham group, the enzyme activity of SOD decreased 6 h after surgery in the CCH group, and there were significant differences at 1W ($p < 0.01$), 2 W ($p < 0.01$) and 4 W ($p < 0.01$) (Figure 3 I).

DNMTs Expression Was Increased In Corpus Callosum Region After CCH

The transcription levels of DNMT1, DNMT3a and DNMT3b in the white matter at different time points after CCH were detected by qRT-PCR. Compared with Sham group, the transcription level of DNMT1 in CCH group was significantly upregulated at 2 W ($p < 0.05$), 4 W ($p < 0.05$), 8 W after surgery ($p < 0.01$). Compared with Sham group, the transcription level of DNMT3a in CCH group was upregulated at 4 W ($p < 0.05$) and 8 W ($p < 0.01$). Compared with Sham group, the transcription level of DNMT3b in CCH group was significantly upregulated at 2 W ($p < 0.01$), 4 W ($p < 0.01$), 8 W ($p < 0.01$) (Figure 3 A-C).

The protein levels of DNMT1, DNMT3a and DNMT3b in the white matter at different time points were detected by Western blotting. Compared with Sham group, the protein level of DNMT1 in CCH group was significantly upregulated at 2 W ($p < 0.01$), 4 W ($p < 0.01$), 8 W after surgery ($p < 0.01$). Compared with Sham group, the protein level of DNMT3a in CCH group was significantly upregulated at 2 W ($p < 0.01$), 4 W ($p < 0.01$), 8 W ($p < 0.05$). Compared with Sham group, the protein level of DNMT3b in CCH group was upregulated at 2 W, 4 W, 8 W, but there was no significant difference ($p > 0.05$) (Figure 3 D-G).

CCH Induced Hypermethylation of A2AR Gene Promoter Region

Methylation of A2AR gene promoter was evaluated by BSP at 4 W after surgery. The non-methylated C base was transformed into T after bisulfite treatment, while the methylated C base remained unchanged. Therefore, the reverse sequencing was based on the principle of base complementation, where the G front represented the methylated state, and the A front represented the non-methylated state. BSP results showed that CCH group had obvious G front at CpG12, while in Sham group this changed to A front (Figure 4 A). The methylation level of each CpG site was compared, and it was found that the average methylation level of CpG12 site in the CCH group was significantly higher than that in the Sham group ($p < 0.01$). No significant differences in methylation levels were detected in the rest CpG sites (Figure 4 B).

Methylation Inhibitor Played a Protective Role in Chronic Cerebral Hypoperfusion by Ameliorating the Downregulation of A2AR

White Matter Lesions After CCH can be Alleviated or Aggravated by Regulating A2AR Activity

White matter lesions were evaluated by Kluver-Barrera staining. Compared with Sham group, nerve fibers of CCH group rats became loose, some fibers were disordered, and local

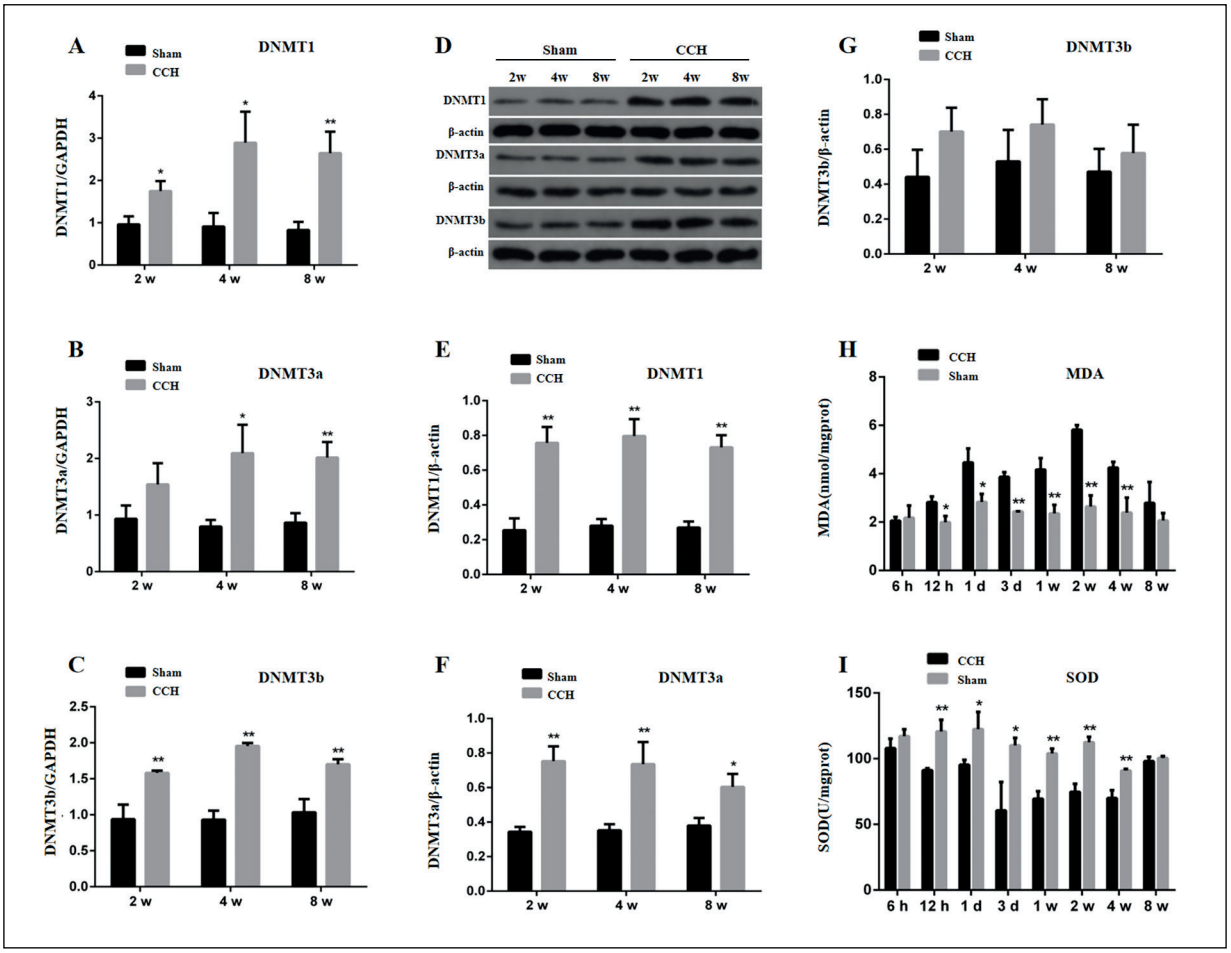

Figure 3. A-C, The mRNA levels of DNMT1, DNMT3a and DNMT3b in the white matter at different time points were measured by qRT-PCR (n = 3). **D-G,** The protein levels of DNMT1, DNMT3a and DNMT3b in the white matter at different time points were detected by Western blotting (n = 3). **H,** The concentration of MDA in the brain tissue was detected by lipid peroxidation MDA assay kit (n = 3). **I,** The enzyme activity of SOD in the brain tissue was detected by total superoxide dismutase assay kit with WST-8 (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

cavitation appeared, and the changes were more obvious with the prolongation of chronic cerebral hypoperfusion. Compared with CCH group, in CCH+A2AR inhibitor group the nerve fibers became looser, with disordered arrangement of fibers and increased vacuoles. Compared with CCH group, in the CCH+A2AR agonist group the nerve fibers became tighter, with reduced disturbance and vacuoles. These results suggested that CCH could cause nerve fibers injury in white matter of the brain, whereas A2AR inhibitor could aggravate nerve fiber injury, and A2AR agonist could alleviate nerve fiber injury under CCH (Figure 2 C).

A2AR protein expression in corpus callosum was evaluated by Western blotting. Compared with CCH group, A2AR protein level in corpus callosum in CCH+A2AR inhibitor group reduced after A2AR inhibitor treatment at 2 W ($p < 0.05$), 4 W ($p < 0.01$), 8 W after surgery ($p < 0.01$). Compared with CCH group, A2AR protein level in corpus callosum in CCH+A2AR agonist group increased after A2AR agonist treatment at 2 W ($p < 0.01$), 4 W ($p < 0.01$), 8 W after surgery ($p < 0.01$) (Figure 4 G-H).

A2AR protein level in serum was evaluated by ELISA. Compared with Sham group, serum A2AR protein level in CCH group significantly reduced at 2 W ($p < 0.01$), 4 W ($p < 0.01$), 8 W ($p < 0.01$). Compared with CCH group, serum A2AR protein level in CCH+A2AR inhibitor group reduced after A2AR inhibitor treatment at 2 W ($p < 0.01$), 4 W ($p < 0.01$), 8 W after surgery ($p < 0.01$). Compared with CCH group, the level of serum A2AR protein in CCH+A2AR agonist group increased after A2AR agonist treatment at

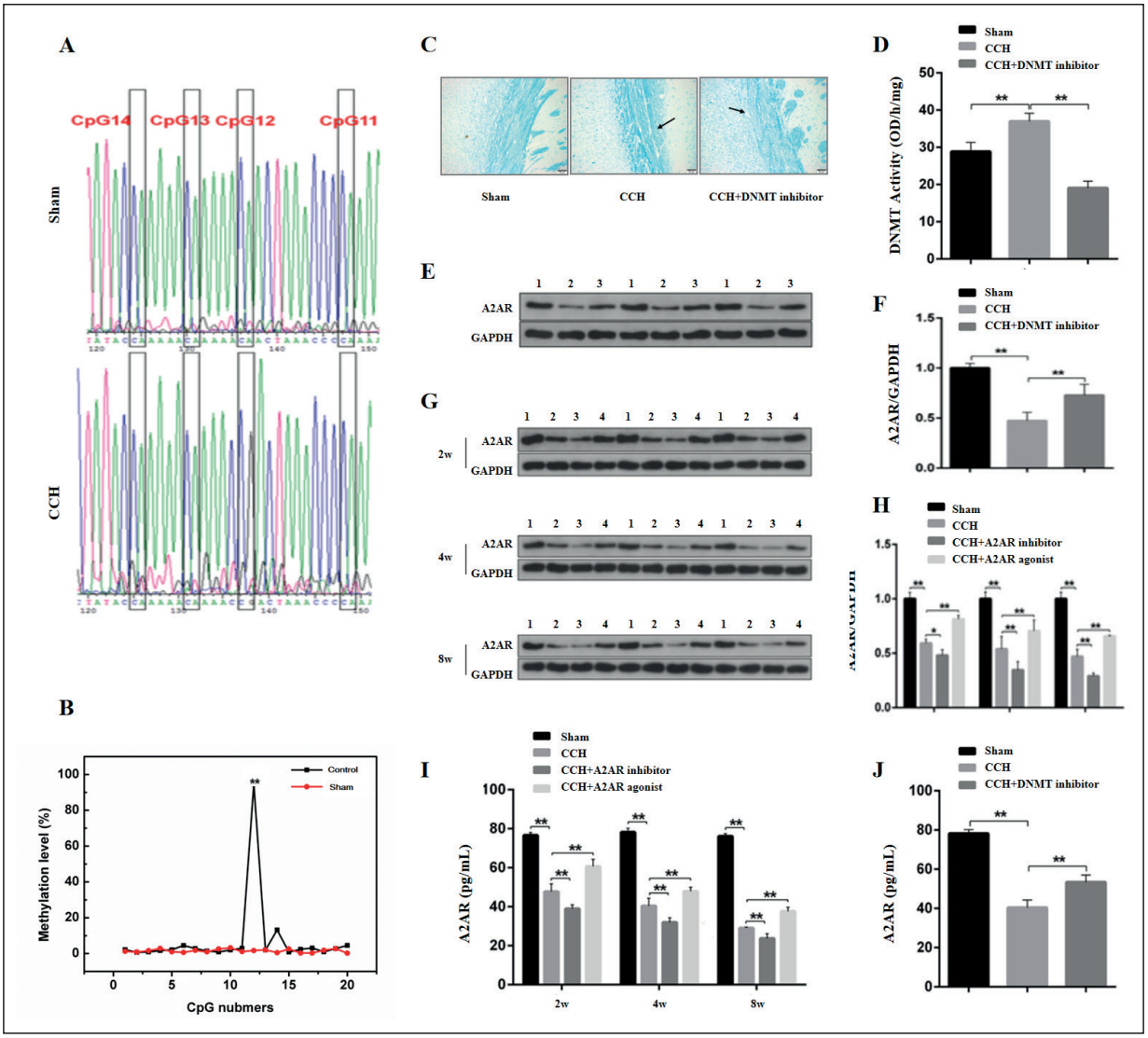

Figure 4. A-B, Methylation of A2AR gene promoter region measured by BSP (n=3). **C,** K-B staining of pathological sections in cerebral white matter. **D,** The enzyme activity of DNA methyltransferase was detected by DNMTs activity assay kit (n = 3). **E-F,** A2AR expression in corpus callosum was detected by Western blotting (1, 2 and 3 represent Sham group, CCH group and CCH+DNMT inhibitor group, respectively) (n = 3). **G-H,** A2AR expression in corpus callosum was detected by Western blotting (1, 2, 3 and 4 represent sham group, CCH group, CCH+A2AR inhibitor group and CCH+A2AR agonist, respectively) (n = 3). **I-J,** A2AR level in serum was detected by ELISA (n = 3) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, scale bars represent 50 μm).

2 W ($p < 0.01$), 4 W ($p < 0.01$), 8 W after surgery ($p < 0.01$) (Figure 4 I).

Methylation Inhibitors Can Rescue the Downregulation of A2AR and White Matter Lesions

The enzyme activity of total DNMTs was detected by the DNMTs activity kit. Compared with the sham group, the activity of total DNMTs in CCH group increased. Compared with CCH group, the activity of total DNMT enzyme in the nucleoprotein of rat corpus callosum significantly decreased at 4 W ($p < 0.01$) after DNMT inhibitor treatment. These results suggested that DNMT inhibitors could reduce the increase of total DNMT enzyme activity of the nucleoprotein in the corpus callosum of rats under chronic hypoperfusion (Figure 4 D).

White matter lesions for each animal were evaluated by Kluver-Barrera staining. Compared with Sham group, nerve fibers of CCH group rats became loose, some fibers were disordered, and local

cavitation appeared, and the changes were more obvious with the prolongation of chronic cerebral hypoperfusion. Compared with CCH group, white matter lesions of CCH+DNMT inhibitor group reduced at 4 W, where the nerve fibers became compact with the degree of disorder and the vacuoles reduced. The results suggested that DNMT inhibitors could reduce the nerve fiber lesions in rats under chronic hypoperfusion (Figure 4 C).

A2AR protein expression in corpus callosum was evaluated by Western blotting. Compared with CCH group, the expression of A2AR protein in corpus callosum in CCH+DNMT inhibitor group increased after DNMT inhibitor treatment at 4 W ($p < 0.01$). The results suggested that DNMT inhibitors could alleviate the decrease of A2AR protein expression in the corpus callosum of rats under chronic hypoperfusion (Figure 4 E-F).

A2AR protein level in serum was evaluated by ELISA. Compared with CCH group, the level of serum A2AR in CCH+DNMT inhibitor group increased after DNMT inhibitor treatment at 4 W ($p < 0.01$). The results suggested that DNMT inhibitors could alleviate the decreased expression of A2AR protein in serum of rats under chronic hypoperfusion (Figure 4 J).

Discussion

The pathophysiological mechanism of IWML is complex, and its pathogenesis remains elusive. Previous clinical and experimental studies[7,12] showed that the typical pathological features of IWML were the sparsity of white matter nerve fibers, myelin lesions, glial cell activation in white matter lesions area, peripheral immune inflammatory cell infiltration, and significantly increased inflammatory mediators like cytokines, indicating that the inflammatory response was closely related to IWML. Adenosine and its receptors have been shown to be pivotal in regulating inflammation[13]. In our previous research[9,14], we have proved that A2AR is involved in the cerebral chronic hypoperfusion white matter lesions *in vivo* and *in vitro*, but the dynamic change of A2AR and its mechanism are still unclear. In this study, we further explored the roles and mechanism of A2AR in white matter lesions in general pathological conditions.

Previous studies[15] suggested that the role of A2AR in cerebral ischemic injury is very complex, showing different effects in different disease models. In the cerebral chronic hypoperfusion model, our results showed that cognitive decline and the A2AR down-regulated expression in white matter lesions were correlated. The specific A2AR agonist and inhibitor could reduce or aggravate the cognitive impairment, respectively. Our results indicate that the downregulated expression of A2AR in CCH mediates white matter lesions and the impairment of cognitive function.

We then explored the mechanism of A2AR downregulation under CCH. Recently, many studies[16] have indicated a link between oxidative stress and epigenetic changes, and various pathological states (such as ischemia and hypoxia) can induce epigenetic modification of genes in cells[17,18]. As the main epigenetic mechanism, DNA methylation regulates gene transcription and expression by changing the degree of methylation in the promoter region of genes[19]. Furthermore, DNA methylation is catalyzed and maintained by DNMTs. The main catalytic agents of mammalian DNMTs are Dnmt1, Dnmt3a and Dnmt3b[20]. A large number of studies[21] have proved that the quantity or activity of DNMTs can be changed under special circumstances, which then lead to changes in DNA methylation level, and ultimately affect the expression of related genes. Therefore, we hypothesized that the DNA methylation mechanism is involved in the downregulation of A2AR expression in cerebral chronic hypoperfusion white matter lesions.

Our results showed that there were differences in the MDA concentration and enzyme activity of SOD between CCH group and Sham group, and there might be gene modification caused by oxidative stress under CCH. Moreover, compared with Sham group, the average gene methylation level in the CCH group was significantly increased at CpG12 ($p < 0.01$), showing a hypermethylation state. Meanwhile, the protein expression of A2AR in the white matter area of CCH group was significantly lower than that in Sham group. This suggests that the downregulation of A2AR in the white matter lesions may be caused by hypermethylation in the promoter of A2AR gene. Our study also showed that the expression of DNA methyltransferases (including DNMT1, DNMT3a and DNMT3b) in the damage area of white matter in the CCH group was upregulated at 2, 4 and 8 weeks after surgery. Our results suggested that oxidative stress leads to gene modification under cerebral chronic hypoperfusion, which increases the expression of DNA methyltransferases in corpus callosum, thus further enhances methylation in the pro-

moter region of A2AR gene, and then, downregulates the expression of A2AR gene.

Subsequently, we used DNMT inhibitor to intervene in CCH model rats. Our results also showed that A2AR protein level in the corpus callosum and peripheral blood serum of the CCH+DNMT inhibitor group was higher than that of CCH group, and the white matter injury was also alleviated compared with the CCH rats. It suggests that DNMT inhibitors may protect white matter by inhibiting the methylation of A2AR promoter region and rescuing the downregulation of A2AR expression in chronic cerebral hypoperfusion.

Conclusions

In summary, our study suggests that oxidative stress induces the up-regulation of DNMTs expression, leading to hypermethylation in the promoter region of A2AR gene and subsequent downregulation of A2AR expression, which results in white matter lesions under chronic cerebral hypoperfusion. DNMT inhibitors may play a protective role against CCH-induced white matter injury. The results of this study may be helpful to reveal the molecular mechanisms of white matter lesions from the perspective of epigenetics and may provide new ideas and target molecules for future prevention and treatment.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding Sources

This work was supported by the National Natural Science Foundation of China (No. 81571129 and No. 82171334).

Authors' Contribution

WH contributed to conception and design of the study. YW and XZ organized the database. YW, CC, and XZ performed the statistical analysis. YW and CC wrote the first draft of the manuscript. WC and XC wrote sections of the manuscript. ZY provided writing guidance and participated in the revision. KD and LZ participated in some experimental operations. All authors contributed to manuscript revision, read, and approved the submitted version.

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