RESEARCH ARTICLE

N-Acetylcysteine Prevents Sleep Deprivation-induced Memory Deficit in Juvenile Rats Through the Suppression of BDNF, Cortisol, Acetylcholine Levels, and Inflammatory Cytokines Expressions

Citra Rosyidah[1,2], Wawaimuli Arozal[3,*], Hee Jae Lee[4], Raymond Rubianto Tjandrawinata[5], Wahyunia Likhayati Septiana[6]

[1]Doctoral Programme in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya 6, Jakarta 10430, Indonesia
[2]Department of Physiology, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan, Makassar 90245, Indonesia
[3]Department of Pharmacology and Therapeutics, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya 6, Jakarta 10430, Indonesia
[4]School of Medicine, Kangwon National University, 1 Gangwondaehak-gil, Chuncheon-si, Gangwon-do 24341, Republic of Korea
[5]Center for Pharmaceutical and Nutraceutical Research and Policy, Universitas Katolik Atma Jaya, Jl. Jendral Sudirman 5, Jakarta 12930, Indonesia
[6]Department of Histology, Faculty of Medicine, Universitas Gunadarma, Depok 16451, Indonesia

*Corresponding author. Email: wawaimuli.arozal@ui.ac.id

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Abstract

BACKGROUND: Sleep deprivation (SD) affects 20–30% of children and is known to impair cognitive functions, particularly memory. Despite its impact, there is currently no standardized treatment. Evidence from both adult animal and human suggests that N-acetylcysteine (NAC) possesses neuroprotective properties. This study was conducted to evaluate the effects of NAC on memory deficits induced by SD in juvenile rats.

METHODS: Juvenile Sprague-Dawley rats were subjected to SD using the modified multiple platform method. NAC was administered intraperitoneally at doses of 100 mg/kgBW or 500 mg/kgBW. Y-maze and novel object recognition (NOR) tests were used for neurobehavioral assessment. Biochemical analyses were conducted to measure cortisol, brain-derived neurotrophic factor (BDNF), and acetylcholine (ACh) levels, using enzyme-linked immunosorbent assay (ELISA). Acetylcholine esterase (AChE) activity was measured by colorimetric method. Western blot analysis was performed to examine cAMP response element-binding protein (CREB) and phosphorylated CREB (p-CREB). Additionally, cytokine mRNA expressions were evaluated using polymerase chain reaction (PCR).

RESULTS: Spontaneous alteration and discrimination ratio were decreased in SD group without treatment compared to the normal group ($p<0.05$). Similarly, BDNF was also decreased compared with normal group ($p<0.05$). Cortisol level and mRNA expression of TNF-α were increased significantly compared with normal group ($p<0.05$) along with a slight increase of ACh activity. Interestingly, NAC treatment mainly at the dose of 500 mg/kgBW prevented those pathological features significantly.

CONCLUSION: NAC might prevent the SD-induced memory deficits by suppressing the inflammatory markers, activity of AChE, cortisol, and enhancing the level of BDNF.

KEYWORDS: SD, NAC, memory, AChE, cortisol, inflammatory markers, BDNF

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Introduction

Children's general well-being, physical performance, and cognitive function largely depend on having sufficient sleep. (1) Twenty to thirty percent of youngsters suffer from sleep deprivation (SD), which can start as early as age six. Getting sufficient sleep during childhood helps children remember phoneme sequences and retain their meanings for extended periods.(2) From a neurological point of view, SD has a

negative impact on children's neurobehavioral performance, particularly memory.(3)

Rapid eye movements, low amplitude, high-frequency electroencephalograph (EEG) waves, and muscular atonia are characteristics of the rapid eye movement (REM) stage of sleep.(4) According to some studies, subjects will endure memory deficits when REM-SD occurs. The exact mechanisms underlying it are still unknown. They might be linked to the alteration in the concentration of several important signaling molecules such as cortisol, acetylcholine esterase (AChE), acetylcholine (ACh), and brain-derived neural growth factor (BDNF), that are involved in memory and synaptic function.(1)

When SD or other stressors occur, cortisol is secreted into the bloodstream and attaches to its intracellular receptors, including those in the brain. Following SD, blood cortisol levels were higher than during regular sleep.(5) Several studies have connected high cortisol levels to memory deficits. The rats' hippocampal and cortical regions showed increased AChE activity after participating in SD experiments.(6,7) As AChE hydrolyzes the ACh, this condition may cause memory impairment because ACh is essential for memory.(8)

It has been demonstrated that SD increases some proinflammatory cytokines, including interleukin (IL)-1β and tumor necrosing factor (TNF)-α, which results in neuroinflammation. Following SD, rats also exhibit an increase in the nucleotide-binding domain and leucine-rich repeat protein-3 (NLRP3) inflammasome, which activates caspase to transform IL-1β into its active form.(9)

BDNF significantly impacts synaptogenesis and synaptic plasticity. After SD procedures, its level is compromised.(10,11) There was also an increase in c-AMP response element-binding protein (CREB). This factor controls the transcription of the BDNF gene, and its phosphorylated version, p-CREB.(12) Nicotinamide adenine dinucleotide phosphate diaphorase (NAPDH) staining can be applied to monitor the reduction in neurogenesis by identifying the group of neurons that are generating nitric oxide synthase (NOS).(13,14)

N-acetylcysteine (NAC) has been proposed for its effect as a neuroprotector on several neurological conditions such as stroke, Alzheimer's, and Parkinson's disease.(15) An experimental study conducted on SD-induced memory deficit in adult rats treated with NAC, proved that NAC has a protective effect against SD-induced cognitive dysfunctions and anxiety-like behavior.(16) Chronic SD in juvenile rats may exhibit anxiety, depression-like behaviour and decrease in BDNF in the adult life.(1) However, to the best of our knowledge, no study performed the use of NAC in juvenile rats undergoing SD in acute setting. Therefore, this study was conducted to determine the protective effect of NAC on neurobehavioral tests, cortisol, ACh, AChE activity, inflammatory cytokines, CREB, p-CREB, BDNF, along with histopathological changes in neuronal density in hippocampus tissue.

Methods

Animals and Grouping

Sprague-Dawley male rats (n=24), 3 weeks, weighing between 40-60 g, were used in this study. These rats were obtained from Bogor Agriculture Institute animal breeding facilities. Rats were placed in closed system animal cages at the Animal Research Facility (ARF), Universitas Indonesia, in a standard room (constant temperature and humidity, adequate light, and 12 h of light and 12 h of darkness) and were given food and drink *ad libitum*. Rats were randomly divided into four groups: normal rats that didn't receive the SD procedure; as well as rats receiving SD procedures and receiving either 0, 100, or 500 mg/kg BW/daily NAC administration. Animals were acclimated for 7 days before experimentation. The detailed of experimental procedures was depicted in Figure 1. The Ethics Committee of the Faculty of Medicine, University of Indonesia approved the experimental protocol of this study (No. KET-562/UN2.F1/ETIK/PPM.00.02/2021).

SD Procedures

A modified multiple-platform method was used to induce REM-SD in rats. This method was used widely to initiate REM-SD without influencing non-REM (NREM) sleep. For 72 hours, two rats were kept in water tanks of 40 × 30 × 18 cm and equipped with four circular platforms 5 cm high. The water in the tanks reached 1 cm below the platforms' surface. Without contact with the water, the rats were placed on the platforms. They could move inside the tank and hop up to the platform. The diameter of the platforms was 10 cm and 5 cm for the normal and test animals (SD groups), respectively. Animal movements during the SD procedures were monitored by a closed-circuit television (CCTV) (Hikvision, Hangzhou, China). This procedure was modified based on previous study.(17)

NAC Administration

The normal group received no SD procedures and no injection, while the SD group received only intraperitoneal

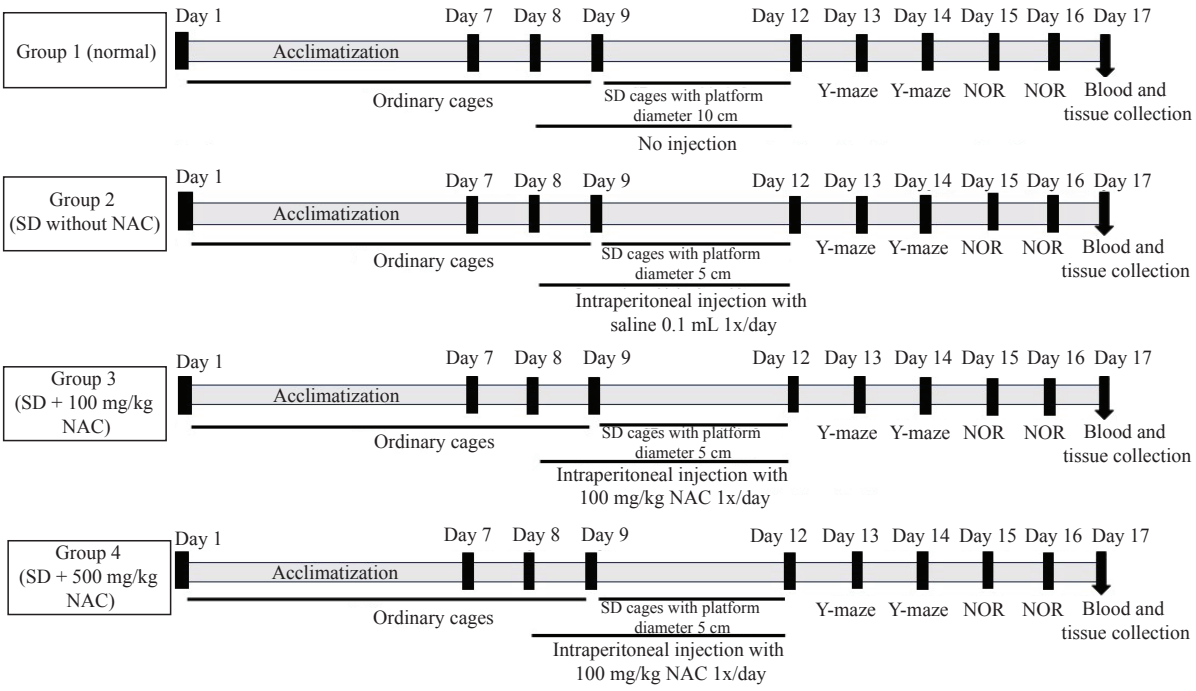

Figure 1. The timeline of the study.

injections of 0.1 mL saline (Otsuka, Malang, Indonesia) once a day during the SD procedures. The third and fourth group received NAC (Dexa Medica Company, Banten, Indonesia) by intraperitoneal injection, with doses of 100 and 500 mg/kg BW/daily, respectively. The NAC as well as the saline were administered in the morning at 9 A.M. The total duration of injections (NAC or saline) was 5 days, *i.e.*, 2 days in closed-system cages (normal cage) and 3 days in cages with multiple platforms (SD procedure).

Y-maze Test

The y-maze test was used to assess spontaneous alternation as a measurement for the spatial working memory of the rats. The test was performed using the method described previously.(18) In brief, the behavior was measured for 10 minutes and recorded using an automatic system from the CCTV. Spontaneous alternation was observed when animals remember the arms previously visited and show a tendency to enter a less recently visited arm. The apparatus for the y-maze test consisted of a black acrylic maze (35 cm long and 7 cm wide, with 15 cm high walls) with three arms and an angle of 120° between the arms. For 15 minutes, rats were placed within an arm, and the amount of arm entries and alternations (*i.e.*, consecutive entry sequences of BAC, CBA, or ABC but not CAC, not ABA, *etc*.) were recorded for each rat. The percentage of spontaneous alternation was calculated using the formula: [(Number of alternations)/(Total arm entries-2)]×100.

Novel Object Recognition (NOR) Test

NOR was a behavioral test assessing animals' attempts to recognize new objects in their environment. It was known as the discrimination index, namely the percentage of time in examining the new object over the entire period spent in examining both the new and old objects. The test was carried out according to the previous reported method (19) with some modifications. This experiment used a white acrylic closed chamber (50 × 80 × 80 cm) with a dim light condition. The familiarization phase was held a day after the Y-maze test. The rats were placed in the chamber without any object for 5 min. The test phase consisted of two steps a day after the familiarization phase. The time for each step is 10 min. In the first step, a rat was presented with two objects 10 cm from the wall. In the second step, one of the objects was replaced with a new one. To measure the recognition memory of the animals, the discrimination index according to the following formula was calculated: time for observing the new object/(time for observing the new object + time for observing the old object).

Blood, Hippocampus, and Prefrontal Cortex (PFC) Collection

After all the neurobehavioral tests had been done, rats were anesthetized intraperitoneally using xylazine hydrochloride (Cat. No. X1251; Sigma-Aldrich, St. Louis, MO, USA) 0.01 mL/kg BW and ketamine (Cat. No. 343099; Sigma-Aldrich) 0.03 mL/kg BW, and blood was collected from the left

ventricle using a 3 mL syringe, then put in a tube containing ethylenediaminetetraacetic acid (EDTA) K3 (Cat. No. E1164; Sigma-Aldrich). Rats were decapitated immediately after the blood collection and the whole brain was extracted. After being removed, the PFC for quantitative real-time polymerase chain reaction (qRT-PCR) and the hippocampal tissue for enzyme-linked immunosorbent assay (ELISA) and western blot were rinsed with cold saline and preserved at -80°C. The hippocampal tissue for histopathological analysis was fixed by using Phosphate-Buffered Saline (PBS) that contain 4% paraformaldehyde (Cat. No. J61899. AK; Thermo Fisher, Waltham, MA, USA). Plasma was extracted after a centrifugation at 4°C, 3000 rpm for 10 min.

Examination of Hippocampal and PFC Protein Levels

After thawed in room temperature, the hippocampal and PFC tissue were then mixed with Tris-buffered saline (TBS) (Cat. No. J62533.AP; Thermo Fisher) and containing protease (Cat. No. A32963.AP; Thermo Fisher) and phosphatase inhibitors (Cat. No. 78420; Thermo Fisher) to preserve protein integrity. The tissue was disrupted using a homogenizer to ensure complete lysis. The homogenate was centrifuged at 15,000 g for 15 minutes at 4°C to remove debris. The Bradford method was used to determine the total protein in the samples.(20)

Measurement of Cortisol, ACh, and BDNF Levels

We measured cortisol from the plasma using an ELISA kit (Cat. No. EIAHCOR; Thermo Fisher). The ACh level in hippocampus tissue was measured using an ELISA kit (Cat. No. EH42RB; Thermo Fisher). The level of BDNF in the hippocampus was checked by BDNF ELISA kit (Cat. No. ab65345; Abcam, Cambridge, UK) according to the product information of those commercial kits. The optical density absorbance readings were performed in a microplate reader.

Measurement of AChE Activity in the Hippocampus

The AChE activity measurement was carried out using a commercial kit (Cat. No. E-EL-R0355; Elabscience, Wuhan, China). This kit used a colorimetric method to observe the activity of the AChE in converting acetylcholine to choline. The 5-mercapto-nitrobenzoic acid, with an absorption peak at 412 nm, was formed from the reaction of choline with dithiol p-nitrobenzoic acid.

Western Blot Analysis for CREB and p-CREB in the Hippocampus

Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, the protein samples from hippocampus tissue were sorted by molecular weight. The procedure was as follow: Equal amounts of protein were mixed with SDS sample buffer and heated at 95°C for 5 minutes to denature the proteins. The resolving and stacking gels were prepared, the gel apparatus was assembled, and running buffer was added. The prepared protein samples and molecular weight markers were loaded into the wells. Electrophoresis was conducted at 100–150 V until the dye front reached the bottom, ensuring effective separation of proteins based on size. Then, they were transferred to a nitrocellulose membrane (Nitrocellulose Membrane Filters, Cat. No. 88018; Thermo Fisher). The membranes were labeled with the first antibody (CREB, p-CREB, and GAPDH as a housekeeping gene, with a concentration of 1:5000) diluted in blocking buffer and left overnight at 4°C after being treated with 5% skim milk in TBS with Tween 20 for 30 minutes. Membranes were placed in secondary antibodies (1:10.000) and diluted in blocking buffers. The enhanced chemiluminescence substrate (Pierce™ ECL Western Blotting Substrate) (Cat. No. 32106; Thermo Fisher) and the Chemiluminescence Alliance 4.7 (Uvitec, Cambridge, UK) were used to identify the signals. The band of the particular target protein was finally displayed using the Gel Documentation Gel Doc™ XR+ System quantification (Cat. No. 1708195; Bio-Rad, Hercules, CA USA). The data was provided in arbitrary units. Antibodies used for Western blot analysis were GAPDH (Cat. No. 14C10; Cell Signaling Technology, Beverly, MA, USA), Rabbit mAb (Cat No. 2118; Cell Signaling Technology), CREB (Cat. No. 48H2; Cell Signaling Technology) Rabbit mAb (Cat. No. 9197; Cell Signaling Technology), p-CREB (Ser 133) (Cat. No. 87G3; Cell Signaling Technology) Rabbit mAb (Cat. No. 9198; Cell Signaling Technology), and Anti Rabbit IgG HRP Link-Antibody (Cat. No. 7074S; Cell Signaling Technology). Western blot allows for the detection of CREB protein levels, providing insights into post-translational modifications such as phosphorylation, which are crucial for CREB's activity.

mRNA Expression for Cytokines in PFC Tissue

Using a total RNA micro kit (Geneaid Biotech, New Taipei, Taiwan) and following the kit's instructions, total RNA was extracted from the PFC tissue. A nanodrop spectrophotometer (260 nm) examined the total RNA amount. The qRT-PCR measurements were performed according to a published protocol.(21) The *IL-1β*, *TNF-α,* and *NLRP3* mRNA expression analysis was performed using qRT-PCR applied Biosystem with ReverTra® qPCR RT Master Mix/gDNA remover kit (Toyobo, Osaka,

Japan). The primer sequences specific to the genes are listed in Table 1. The *β-actin* was used as the housekeeping gene. The total RNA was collected using Direct-zol RNA Miniprep Plus (Cat. No. R2073; Zymo Research, Irvine, CA, USA). ReverTra AceTM qPCR RT Master Mix with gDNA Remover (Cat. No. FSQ-301; Toyobo) and qRT-PCR (MiniOpticonTM Real-Time PCR System, Bio-Rad) were then used to reverse-transcribe the RNA into cDNA. The THUNDERBIRDTM SYBR® qPCR Mix (Cat No. QPS-201; Toyobo) was used for the procedure. The qRT-PCR cycle program was started at 95°C for two minutes, then it went through 40 cycles of denaturation at 95°C for five seconds, annealing at 60°C for ten seconds, and extension at 72°C for twenty seconds. The Livak method was used to determine each gene's relative expression.(22)

NADPH Diaphorase Staining

Using Leica CM1950 Cryostat (Cat. No. CM1950; Thermo Fisher), sections with a thickness of 20 µm were created and then placed on glass slides (Cat. No. S1111; Matsunami Glass, Osaka, Japan). The slices were incubated for 30 to 60 minutes at 37°C in a solution containing 0.3 mg/mL nitroblue tetrazolium (NBT) (Cat. No. N6876; Sigma-Aldrich) and 0.1 mg/mL NADPH (Cat. No. 10107777001; Roche Diagnostics, Mannheim, Germany) in 0.1 M Tris-HCl buffer (pH 8.0) to perform NADPH labeling. The slides were rinsed in PBS to halt the reaction after incubation and dehydrated in graded ethanol.(14,23) Olympus CX23 (Olympus Corporation, Tokyo, Japan) was used for the observation, and the descriptive analysis for microscopic evaluation was performed by blinded manner.

Statistical Analysis

Data results were presented as the mean±SEM and analyzed using SPSS version 26 (IBM Corp, Armonk, NY, USA), specifically one-way ANOVA followed by Bonferroni's or Tamhane's post hoc test. A value of $p<0.05$ was considered statistically significant. Graphs were presented using GraphPad Prism 9.5 (GraphPad Software, San Diego, CA, USA USA). For histopathological analysis, descriptive analysis was used.(13)

Table 1. Primer Sequences used in qRT-PCR.

Genes		Sequences
IL-1β	Forward	CACCTCTCAAGCAGAGCACAG
	Reverse	GGGTTCCATGGTGAAGTCAAC
TNF-α	Forward	AAATGGGCTCCCTCTCATCAGTT
	Reverse	TCTGCTTGGTGGTTTGCTACGAC
NLRP3	Forward	GTCCAGTGTGTTTTCCCAGAC
	Reverse	TTGAGAAGAGACCTCGGCAG
β-actin	Forward	TGCTGTCCCTGTATGCCTCTG
	Reverse	GTATGTCACGCACGATTTCCT

Results

The Effects of NAC in Juvenile SD Rats on Memory Deficit Assessed by Y-maze and NOR Tests

The results of this study revealed a significant reduction in the percentage of spontaneous alternation in the sleep-deprived (SD) group compared to the normal group (44.1% *vs.* 61.2%; $p<0.05$). Interestingly, NAC treatment demonstrated significant improvements. Specifically, the percentage of spontaneous alternation increased significantly ($p<0.05$, Figure 2A) in the groups receiving 100 mg/kg compared to SD group (70.4% *vs.* 44.1%) and in the group receiving 500 mg/kg NAC compared to the SD group (76.4 % *vs.* 44.1%).

The NOR test was conducted to assess short-term memory. The discrimination ratio of the SD group showed a significant decline compared to the normal group (0.58 *vs.* 0.78; $p<0.05$). In contrast, as shown in Figure 2B, groups treated with NAC displayed significantly increased discrimination ratios, with improvements noted in the 100 mg/kg and 500 mg/kg NAC-treated groups compared to the SD group (0.77 *vs.* 0.74 *vs.* 0.58; respectively, $p<0.05$).

The Effects of Cortisol Level, AChE Activity, and ACh Level in Juvenile SD Rats Treated with NAC

Figure 3A showed that plasma cortisol levels were significantly elevated in the SD group compared to the normal group (0.99 ng/mL *vs*. 0.48 ng/mL; $p<0.05$). NAC administration resulted in a significant decrease of cortisol levels ($p<0.05$) in the dose of 100 mg/kg (0.72 ng/mL *vs.* 0.99 ng/mL) and 500 mg/kg NAC-treated groups compared to the SD group (0.58 ng/mL *vs.* 0.99 ng/mL).

The activity of AChE in hippocampus tissue was increased in the SD group compared with normal group, although the value was not statistically significant (0.85 mU/mg *vs.* 0.66 mU/mg). Treatment with NAC showed a significant reduction of AChE activity in the dose of 500 mg/kg compared with the SD group (0.45 mU/mg *vs.* 0.85 mU/mg; $p<0.05$) (Figure 3B). Similarly, hippocampal ACh level demonstrated a downward trend in the SD group compared to the normal group (13.8 nmol/mg *vs.* 15.1 nmol/mg), while NAC treatment resulted in a dose-dependent

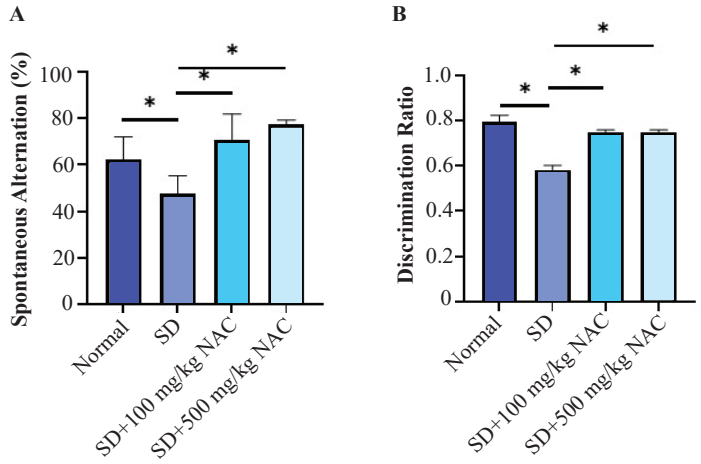

Figure 2. The effects of NAC in juvenile SD rats on memory deficit assessed by y-maze and NOR Tests. A: The effects of NAC in juvenile SD rats on memory deficit in the y-maze test assessed by the percentage alteration. B: The effects of NAC in juvenile SD rats on memory deficit in NOR test assessed by the discrimination ratio. Each column represents mean±SEM of six rats. *Significant if $p<0.05$.

increase in ACh levels (Figure 3C). Nevertheless, this result was not statistically significant.

The Effects of *IL-1β*, *TNF-α*, and *NLRP3* on Juvenile SD Rats Treated with NAC

The mRNA expression of *IL-1β* in the PFC showed a rising trend in the SD group compared to the normal group. In comparison with the SD group, NAC-treated groups (100 mg/kg and 500 mg/kg) showed a tendency to decline the mRNA expression of *IL-1β* (Figure 4A). A similar result was also observed in the result of the mRNA expression of *NLRP3* (Figure 4C). In addition, *TNF-α* mRNA expression significantly increased in the SD group compared to the normal group (6.62 *vs.* 1.09; $p<0.05$). A decreasing trend was observed with 100 mg/kg NAC, and a significant reduction was observed in the 500 mg/kg NAC group compared to the SD group (6.62 *vs*. 1.81; $p<0.05$, Figure 4B).

The Effect of the p-CREB/CREB Protein Expression Ratio on Juvenile SD Rats Treated with NAC

Figure 5A showed the representative band in each group for GAPDH, CREB and p-CREB protein in hippocampus tissue assessed by western blot. Hippocampal protein analysis of CREB/GAPDH, p-CREB/GAPDH, and p-CREB/CREB showed no statistical differences among groups (Figure 5B-5D). Of note, there was a tendency to decrease in SD group compare with N group in terms of p-CREB/CREB ratio, while rats treated with NAC 100 mg tends to ameliorated those changes.

The Effect of the BDNF Level on Juvenile SD Rats Treated with NAC

Figure 6 showed that BDNF concentrations in the hippocampus were significantly reduced in the SD group compared to the normal group (789 pg/mg protein *vs.* 1,325 pg/mg protein; $p<0.05$). NAC treatment significantly increased BDNF levels compared to the SD group ($p<0.05$), at 100 mg/kg (1,722 pg/mg protein *vs.* 789 pg/mg; $p<0.05$) and 500 mg/kg (1,801 pg/mg protein *vs.* 789 pg/mg protein).

NADPH Diaphorase Staining

By descriptive analysis, we observed that NADPH diaphorase staining revealed the highest hippocampal neuronal density in the normal group, which decreased in

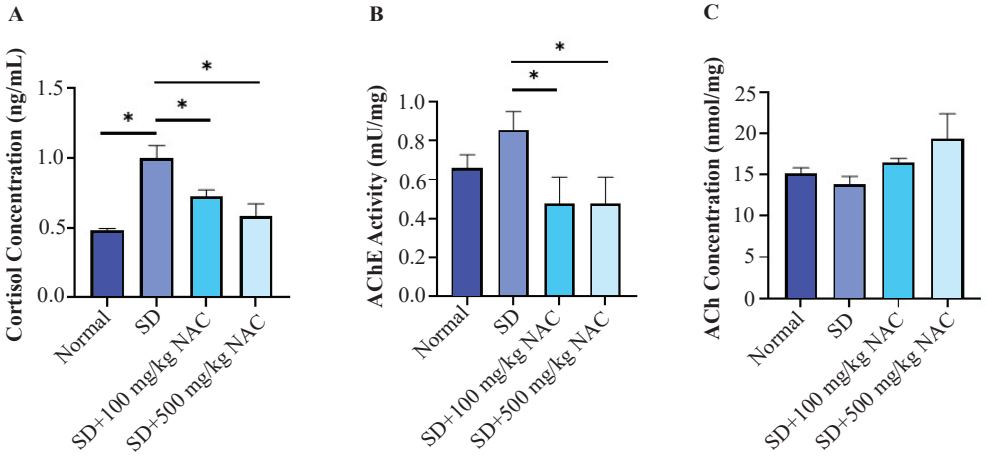

Figure 3. The effects of cortisol level, AChE activity, and ACh level in juvenile SD rats treated with NAC. A: The effects of NAC in juvenile SD rats on memory deficit in the cortisol level. B: The effects of NAC in juvenile SD rats on memory deficit in AChE activity. C: The effects of NAC in juvenile SD rats on memory deficit in the ACh level. Each column represents mean±SEM of six rats. *Significant if $p<0.05$.

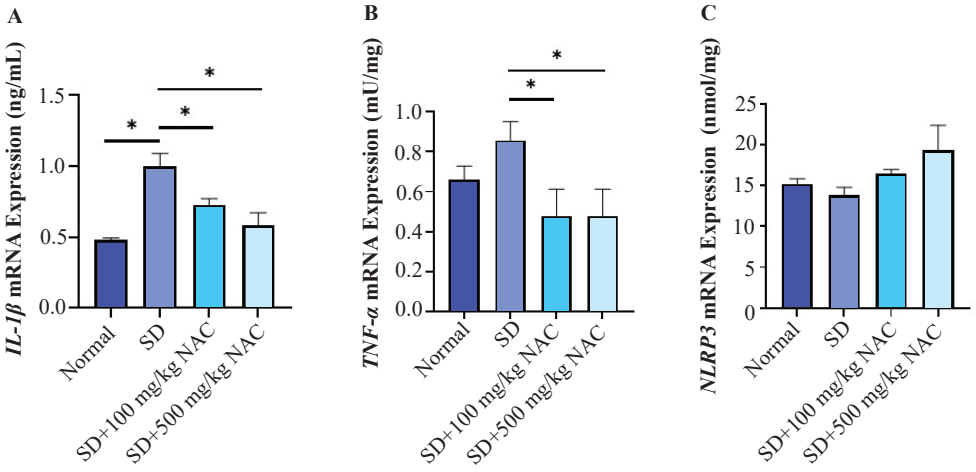

Figure 4. The effects of IL-1β, TNF-α, and NLRP3 on juvenile SD rats treated with NAC. A: The effects of NAC in juvenile SD rats on memory deficit in the IL-1β. B: The effects of NAC in juvenile SD rats on memory deficit in the TNF-α. C: The effects of NAC in juvenile SD rats on memory deficit in the NLRP3. Each column represents mean±SEM of six rats. *Significant if $p<0.05$.

the SD group. NAC administration restored the neuronal density, both at the dose of 100 mg/kg and 500 mg/kg (Figure 7A-7D).

Discussion

The result of this study showed some convincing evidences that NAC could counteract the detrimental effects of SD, particularly improved the memory performance, increased neuroplasticity, inhibited inflammation in brain caused by SD induction, and preserve the cholinergic system.

In this study, to obtain juvenile rats subjected to SD acute condition, the rats were placed on the SD multiple platform method for a total of 72 hours. Furthermore, to determine the memory function due to SD treatment, behavioral tests were carried out by using of Y-maze and NOR tests. For the y-maze, spontaneous alternation that occurs can be used to measure short-term spatial memory in rodents.(12) A low discrimination ratio in NOR describes low sensitivity or

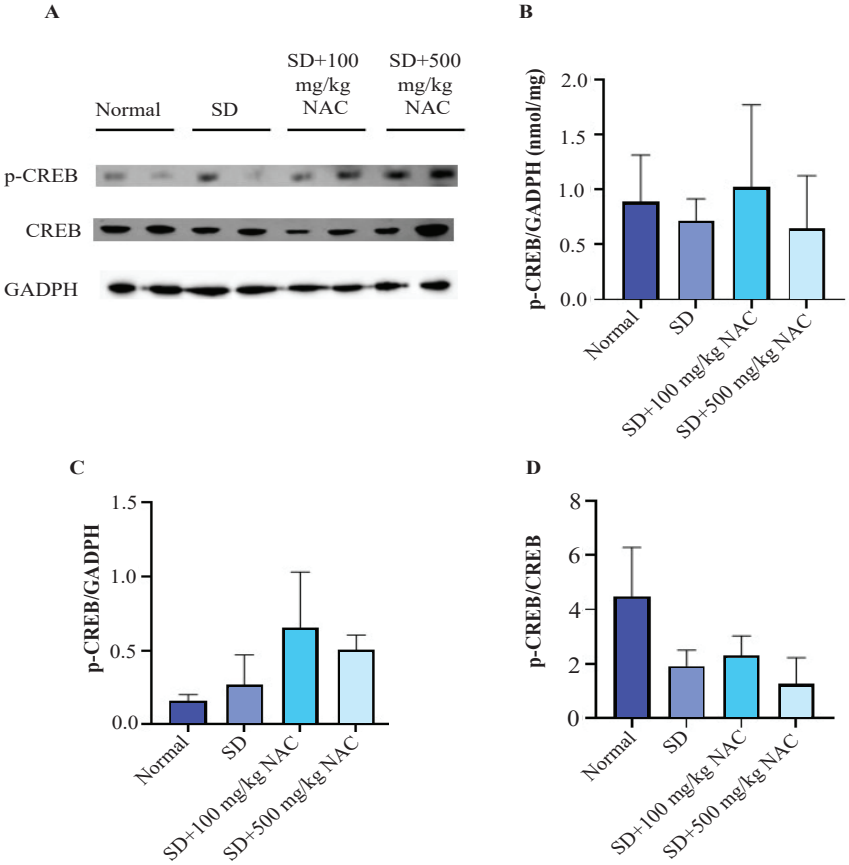

Figure 5. The effect of the p-CREB/CREB protein expression ratio on juvenile SD rats treated with NAC. A: Representative western blot gel in the hippocampus tissue of p-CREB, CREB, and GAPDH. B: The effects of NAC in juvenile SD rats on memory deficit in the CREB/GAPDH ratio. C: The effects of NAC in juvenile SD rats on memory deficit in the pCREB/GAPDH ratio. D: The effects of NAC in juvenile SD rats on memory deficit in the pCREB/CREB ratio. Each column represents the mean ratio±SEM of six rats. *Significant if $p<0.05$.

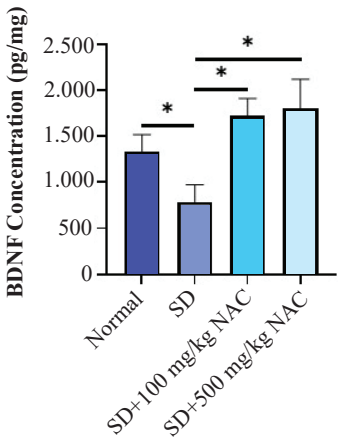

Figure 6. The effects of NAC in juvenile SD rats on memory deficit in the BDNF level. Each column represents mean±SEM of six rats. *Significant if $p<0.05$.

ability to recognize new objects and old objects.(24) The results of this study proved that SD induction significantly reduce memory compared to the normal group, as measured by a decrease in spontaneous alternation and discrimination ratio as shown in Figure 1A and 1B. These results are in line with several previous studies that found that SD procedure causes memory loss in rats.(25,26) SD leads to increased activity in dopaminergic neurons, particularly those in the ventral tegmental area (VTA), that will lead to increased locomotion (27), but in this current study these parameters were not examined. It will be interesting to perform the involvement of this neurotransmitter in the future study.

The purpose of NAC administration was to analyze whether it could improve impaired memory function caused by SD since previous study showed a beneficial effect of NAC in rats with SD procedure by ameliorated the oxidative stress parameters.(16) In this study, the result of y-maze test showed that NAC administration at two different dosages (100 mg/kgBW and 500 mg/kgBW) resulted in a significant increase in the percentage of spontaneous alternation when compared to the SD group, with the 500 mg/kgBW dose providing slightly better results than the 100 mg/kgBW dose (Figure 1A). This result was in line with the NOR test. The current results are similar with other previously conducted studies which found that NAC treatment can improve memory function in experimental animals.(16,28)

SD is a stressful condition that will be detected by the hypothalamic-pituitary axis (HPA) by the paraventricular nucleus (PVN) in the hypothalamus, resulting in increased production of corticotropic releasing factor (CRF) in the organ.(12) CRF subsequently induces the release of adrenocorticotropic hormone (ACTH) in the anterior pituitary which stimulates the adrenal glands to release cortisol into the bloodstream.(29) Current study's results revealed that SD procedure triggered a significant increase in cortisol in the juvenile rats. Intraperitoneal treatment of NAC exhibited a significant reduction of cortisol plasma level in juvenile rats treated with 100 mg/kg and 500 mg/kg (Figure 2A). The reduction of cortisol levels could be attributed to NAC's ability to counteract the increase in free radicals, specifically reactive oxidant species (ROS). As a result, decreasing ROS will lessen the stimulus to the PVN in the HPA.(5,30)

The links between ACh level, AChE activity, and NAC in the context of memory dysfunction caused by SD

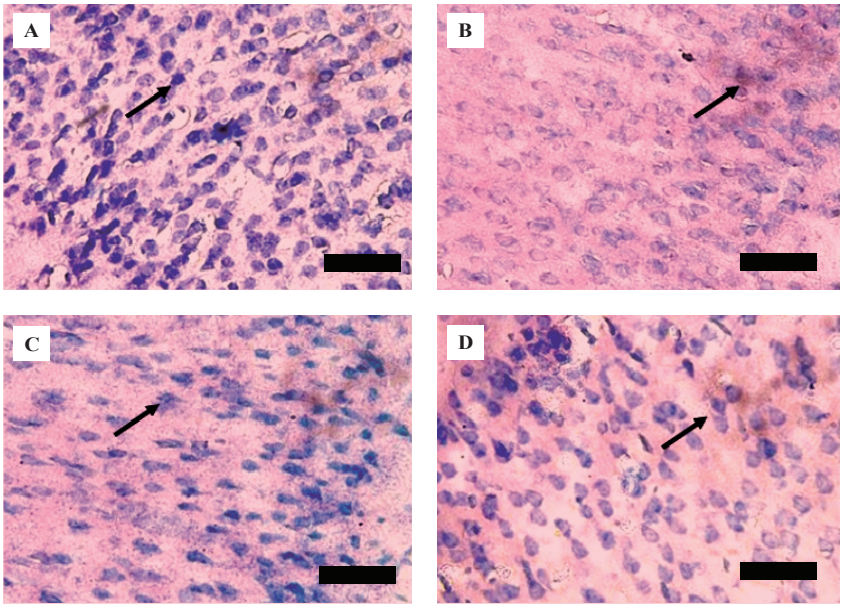

Figure 7. NADPH diaphorase staining of hippocampal tissue. A: normal group; B. SD group; C. SD + 100 mg/kgBW NAC group; D. SD + 500 mg/kgBW NAC group. The black arrows indicate cells that are positively stained with NADPH diaphorase staining. More intense NADPH diaphorase staining generally indicates higher levels of NOS in the cells. Black bars: 50 µm.

procedure were also investigated in this study. ACh and AChE activity are two important elements of cholinergic neurotransmission that influence memory functions. SD have a deleterious effect on the cholinergic system, which can disrupt memory and learning abilities.(31)

The administration of NAC in the current study was found to have a neuroprotective effect by reducing AChE activity along with enhancing ACh level, which might be responsible for the capability to improve memory deficits. (8) NAC acts as an antioxidant, reducing oxidative stress, which helps in helps in regulating the cholinergic system by inactivating AChE and thus blocking ACh breakdown. (31) The lack of performing the oxidative stress status in the current study is considered a limitation of this study.

It was already known that SD causes brain inflammation by upregulating some pro-inflammatory cytokines.(4) In this study, the SD procedure could elevate the expression of *TNF-α* in the PFC, but not in *IL-1β* expression. These discrepancies could be explained as follows: TNF-α is often produced more rapidly in the early phase of the inflammatory response. TNF-α production begins with the recognition of inflammatory stimuli by pattern recognition receptors, which triggers the activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which in turn increase *TNF-α* gene expression. IL-1β, although also produced in the inflammatory phase, takes longer to be produced. IL-1β is produced in an inactive form as pro-IL-1β and is then processed into the active form by the caspase-1 enzyme which is usually activated via the inflammasome (mainly NLRP3).(32,33)

In this study, it was found that the mRNA expression of *NLRP3* also tended to increase in SD group compared to the normal group, suggesting that inflammation is an important pathway mediating SD-induced pathological condition. Interestingly, this study found that administering NAC at the dose of 500 mg/kgBW significantly reduced the mRNA expression of *TNF-α*. Of note, the mRNA expression of *IL-1β* and *NLRP3* in NAC-treated rats tended to decrease compared with SD-only rats. In this study, mRNA analysis for cytokines was performed as also performed by other studies.(34,35) It also will be interesting to check the level of cytokines in blood and PFC by ELISA, since the cytokines levels can be observed systematically in this animal model.

The band intensity of p-CREB in the hippocampal tissue of the SD group was reduced even though the quantification analysis was not statistically significant. Western blot allows for the detection of CREB protein levels, providing insights into post-translational modifications such as phosphorylation in p-CREB, which are crucial for CREB's activity. This tendency to decrease in p-CREB implied that SD interferes with the CREB signaling pathway, which has an impact on the diminished ability to facilitate neuroplasticity alterations, therefore aggravating the cognitive impairment caused by SD.(36) In this study, NAC administration at the dose of 100 mg/kg was demonstrated to tend to increase the band intensity of p-CREB. It can be stated that NAC impacts CREB phosphorylation, therefore improving the function of the CREB signaling system and increasing p-CREB expression.(37,38)

Decreased BDNF as found in this study are associated with synaptic dysfunction and impaired neuroplasticity which can lead to decreased memory function and cognitive abilities. This decreased BDNF level is associated with decreased p-CREB which is a transcription factor of BDNF. (1) The administration of NAC at the dose of 100 and 500 mg/kgBW showed an increase in BDNF levels and as a consequence improved the memory function. In an animal model, NAC could reduce oxidative stress which ultimately helps increase BDNF levels in the brain.(39,40)

The result shown in Figure 7 was consistent with previous studies showing that SD increases oxidative stress and decreases NOS activity, which contributes to neuronal damage.(14) NO produced by NOS plays an important role in neuroplasticity and memory processes. However, in SD conditions, excessive NO production can cause neurodegeneration through the formation of ROS.(13) NAC administration in this study was shown to be able to improve the NADPH diaphorase activity by increasing the neuronal density in the hippocampus. NAC, which functions as a powerful antioxidant, reduces oxidative stress and inflammation by neutralizing ROS.

Since the duration of SD procedure in this studies was only observed for acute condition, further studies are needed to be performed in the chronic setting. In addition, since in this study the animal was treated via intraperitoneal administration to achieve the fast response, further study needed to be conducted using NAC via oral administration.

Conclusion

This study demonstrates that NAC ameliorates SD-induced memory deficits in juvenile rats through a complex mechanism. NAC effectively suppressed inflammatory responses, stabilized cholinergic pathways, decreased cortisol, inhibited the pro-inflammatory cytokine, enhanced neuroplasticity marker BDNF, and reduced AChE

activity. These findings emphazised NAC's potential as a neuroprotective agent for addressing SD-induced memory deficit in juvenile rats.

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Authors Contribution

CR, WA, and HJL participated in the study's conceptualization, planning, and data collection. CR and WLS analyzed the experimental data. WA, HJL, and RRT assisted CR in analyzing and interpreting the results. CR and WA drafted the manuscript and prepared all the figures. All authors participated in the critical review and modification of the manuscript.

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