Therapeutic Effects of Hydrogen Gas Inhalation on Trimethyltin-induced Neurotoxicity and Cognitive Impairment in C57BL/6 Mice Model

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Therapeutic Effects of Hydrogen Gas Inhalation on Trimethyltin-Induced Neurotoxicity and Cognitive Impairment in C57BL/6 Mice Model

Directed by Prof. Kyu-Jae Lee

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This certifies that the Doctoral Dissertation of Eun-Sook Jeong is approved

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I would like to express my gratitude as I finish my Ph.D. program, which started with both excitement and fear of the new challenges it would bring. There are still many shortcomings, and things that I wish I had done differently. However, it was a valuable time that taught me patience, perseverance, a willingness to embrace challenges, passion, and an appreciation for academic studies.

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I would like to express my gratitude to my family and friends who supported me. And I send my love to my most precious son who has always shown encouragement and devoted love by my side.

December 2021
Eun-Sook Jeong

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ABBREVIATIONS

Aβ	β amyloid
AD	Alzheimer's Disease
Apo-E	Apolipoprotein E
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic
Ca^{2+}	Calcium
CNS	Central nervous system
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
G-CSF	Granulocyte colony-stimulating factors
GPx	Glutathione peroxidase assay
H_2	Molecular hydrogen
IP	Intraperitoneal
IL	Interleukin
LC	Lithium chloride
MDA	Malondialdehyde
NFT	Neurofibrillary tangles
NC	Non treated normal control group

NO	Nitric oxide
OS	Oxidative stress
O_2^-	Superoxide
PC	Positive control
p-tau	Phospho-tau
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SEM	Mean ± standard error of the mean
TMT Only	Only TMT injection group
TMT	Trimethyltin
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
1×TBST	1×Tris buffered saline and tween

ABSTRACT

Background: Oxidative stress (OS) is one of the causative factors in the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease (AD) and cognitive dysfunction. In the present study, we investigated the effects of hydrogen (H_2) gas inhalation in trimethyltin (TMT)-induced neurotoxicity and cognitive dysfunction in the C57BL/6 mice.

Methods: First, mice were divided into the following groups: mice without TMT injection (NC), only TMT injection group (TMT Only), TMT injection + lithium chloride treated group as a positive control (PC), and TMT injection + 2% H_2 inhalation treated group (H_2). The TMT injection groups were administered a single dosage of intraperitoneal TMT injection 2.6 mg/kg body weight and H_2 group was treated with 2% H_2 for 30 min once a day for 4 wks. Additionally, the behavioral test was performed with Y-maze to test the cognitive abilities of the mice. Furthermore, multiple OS and AD-related biomarkers such as reactive oxygen species (ROS), nitric oxide (NO), calcium ion (Ca^{2+}), malondialdehyde (MDA), glutathione peroxidase (GPx), catalase, inflammatory cytokines, apolipoprotein (Apo)-E, amyloid β (Aβ)-40, phospho (p)-tau, B-cell lymphoma (Bcl)-2 and Bcl-2-

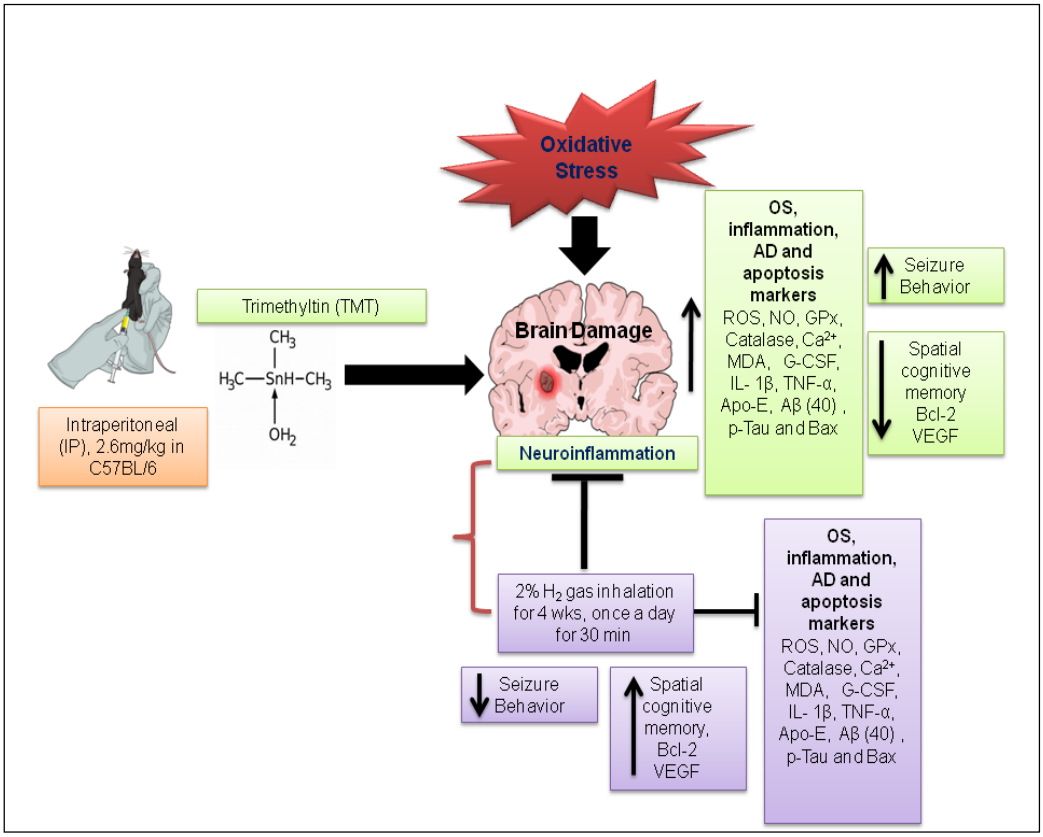
associated X (Bax) were investigated in the blood and brain.

Results: Our results demonstrated that TMT exposure alters seizure and spatial recognition memory. However, after H_2 treatment, memory deficits were ameliorated. H_2 treatment also decreased AD-related biomarkers, such as Apo-E, Aβ-40, p-tau, Bax and OS markers such as ROS, NO, Ca^{2+}, and MDA in both serum and brain. In contrast, catalase and GPx activities were significantly increased in the TMT group and decreased after H_2 gas treatment in serum and brain. In addition, inflammatory cytokines such as granulocyte colony-stimulating factors (G-CSF), interleukin (IL)-6, and tumor necrosis factor (TNF)-α were found to be significantly decreased after H_2 treatment in both serum and brain lysates. In contrast, Bcl-2 and vascular endothelial growth factor (VEGF) expression levels were found to be enhanced after H_2 treatment. Taken together, our results demonstrated that 2% H_2 gas inhalation in TMT-treated mice exhibits memory enhancing activity and decreases the AD, OS, and inflammatory-related markers.

Conclusion: Therefore, H_2 might be a candidate for repairing neurodegenerative diseases with cognitive dysfunction. However, further mechanistic studies are needed to fully clarify the effects of H_2 inhalation on TMT-induced neurotoxicity and cognitive dysfunction.

Keywords: molecular hydrogen, trimethyltin, oxidative stress, Alzheimer's disease,

cognitive dysfunction



[Graphical Abstract]

Therapeutic Effects of Hydrogen Gas Inhalation on Trimethyltin-induced Neurotoxicity and Cognitive Impairment in C57BL/6 Mice Model

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Directed by Professor Kyu-Jae Lee

I. INTRODUCTION

Neurodegenerative diseases are groups of different aging disorders such as Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease which gradually leads to an increase in neuronal cells, accumulation of abnormally aggregated proteins and apoptosis ultimately affecting the motor and cognitive function of an individual [1]. AD is most extensively reported neurodegenerative disease till date and is associated with complex and multifactorial pathophysiology [2, 3]. Due to the continuous increase number of

cases all over the globe, AD has become a major public health problem [4]. Although, there are several factors associated with the development of AD, amyloid β (Aβ) and tau protein are most closely associated to its pathogenesis [4-6]. Aβ abnormally accumulates in AD brain tissues and forms extracellular plaques which are known to induce synaptic alterations and neurodegeneration and may contribute to cognitive deficit. Among Aβ polypeptides in AD condition, majority of amyloid plaques are Aβ-40 and Aβ-42. One of the studies has reported that Aβ-40 type is more abundant form which account for approximately 90% of total Aβ[4].On the other hand, hyperphosphorylation of tau protein forms intracellular neurofibrillary tangles (NFT) and is markedly responsible for neurodegeneration [5, 6]. NFT directly associate with progressive decline in dementia scores and development of cognitive deficits, resulting phosphorylated tau (p-tau) which is a potential player of neurotoxicity in AD [5]. Additionally, studies have shown that multi-site phosphorylation of tau protein can lead to misfolding and aggregation due to conformational changes. In experimental condition of tau pseudophosphorylation at Ser 404, Ser 396, Thr 205 and Ser202 can led to induction of a pathological conformation that are responsible to aggregation [5, 6]. In addition, growing number of evidences have shown that oxidative stress (OS) plays a significant role in progression of neurodegenerative diseases and neurodegeneration, as it contributes to destruction in membrane, cytoskeleton alterations, and apoptosis due

to excessive production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and inflammatory mediators such as inflammatory cytokines [1, 7-10]. In AD, due to oxidative imbalance-mediated injury to the brain region, lipid peroxidation markers are found to be elevated and extensively studied in its pathogenesis [9]. Therefore, OS may easily cause degeneration in the hippocampal region in brain, which is the major region for memory processing [11, 12]. Thus, to ameliorate the neurodegenerative diseases like AD, different therapeutics approaches are developing at rapid pace. Therefore, several animal models, such as trimethyltin (TMT)-induced neurodegeneration are often used for exploring the effects of therapeutic candidates on neurodegenerative diseases like AD.

TMT is a neurotoxin and organometallic compound that induces substantial neurodegeneration and neuronal cell death in central nervous system (CNS), particularly in the hippocampus region in the brain [13]. Several studies have shown that the systemic administration of TMT in human and mice demonstrated clinical symptoms, such as hyperactivity, aggressiveness, cognitive deficits, and seizure-like behaviors [13-15]. However, evidences have shown that all these symptoms are associated with AD [2, 6]. Additionally, previous conducted experimental observations have suggested that TMT-induction induces neuronal cell death, intracellular calcium ion (Ca^{2+}) overload, mitochondrial damage, and OS [16-18]. Moreover, TMT-induction enhances neurogenesis in the brain and increases the

expression of amyloid precursor protein, several signaling pathways, inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and nitric oxide (NO) levels in the hippocampus region, and might be involved in brain injury [19-22]. Due to this reason, TMT-induced animal model is widely used in research area for investigating the brain dysfunction and neurodegeneration [11, 23]. From these background studies, it is important to find an excellent multi-potent agent possessing antioxidant and anti-inflammatory properties for repairing the brain cells against TMT-induced neurotoxicity and cognitive impairment.

Molecular hydrogen (H_2) is potential novel antioxidant and previous published studies have strongly suggested its potential for preventive and therapeutic applications [24-26]. In recent years, research on H_2 therapy for neurodegenerative diseases like AD has become a hotspot due to its anti-oxidative, anti-apoptotic, and anti-inflammatory effects [25, 27]. So far, several animal experiments and more than 25 double blinded clinical studies have been reported regarding the efficacies of H_2 [25, 26]. Numerous routes of H_2administration in body has been reported among which inhalation of H_2 gas, drinking H_2 dissolved water and injection of H_2 dissolved saline are widely studied [26]. Reports have reported that among various administration routes inhalation of H_2 gas is the easiest and simplest method [25, 26]. Administration of H_2 gas by inhalation can readily penetrate through biomembranes, such as the blood-brain barrier (BBB) and the placental barrier

owing to its small molecular size, non-ionic state, and hydrophobic properties, thus benefiting complex organs such as the brain and organelles [28, 29]. Several studies have reported that H_2 plays a positive role in neuroprotection in conditions such as cerebral infarction, Parkinson's disease, cognitive impairment, and brain injury by attenuating the OS, and inflammatory response [26, 28, 30-34]. In previous study conducted using inflammatory disease model, the administration of H_2 showed anti-inflammatory response by ameliorating the pro-inflammatory markers, such as TNF-α, IL-1β, and IL-6 [26, 30, 32]. In addition, Iuchi and colleagues demonstrated that administration of H_2 (approximately 1%, v/v) modulate Ca^{2+} signal and modified the generation of oxidized phospholipids species [35]. Recently, Zhou and colleagues reported that hydrogen rich water has potential to ameliorate TMT-induced memory impairment by regulation of Siah E3 ubiquitin protein ligase 1 [36]. However, the therapeutic effects of H_2 on chemical induced neurodegenerative diseases model such as TMT-induced animal model are poorly studied. Consequently, the actual molecular mechanism through which H_2 treatment induces CNS protection against TMT-induced neurotoxicity and cognitive dysfunction has not been yet fully elucidated. Therefore, based on neurodegenerative and oxidative damage of TMT, and the antioxidative and anti-inflammatory characteristics of hydrogen, the present study aimed to investigate the therapeutic potential effects of 2% H_2 gas inhalation on TMT-induced neurotoxicity

and cognitive impairment in TMT-induced neurodegenerative mouse model. In addition, we explored the effects of H_2 in TMT-induced oxidative and inflammatory response in serum and brain lysates.

II. MATERIALS AND METHODS

2.1. Experimental Animals

Twenty-four male C57BL/6 mice (20-24 g, 8-9 wk old) were purchased from Orient Bio Inc. (Seongnam, Korea). The mice were kept in a pathogen-free environment (22 ± 2 ℃ with 50 ± 10% humidity) under a 12-h light/dark cycle. The mice were given rodent chow and filtered water ad libitum until the end of the experiment. The mice were acclimatized for 10 days in plastic cages (390 × 275 × 175 mm) with wood shaving bedding and were randomly divided into 4 different groups (n=6 per group): non treated normal control group (NC), only TMT injection group (TMT Only), TMT injection + lithium chloride (LC) treated group as a positive control (PC), and TMT injection + 2% H_2 inhalation treated group (H_2). All experimental procedures were performed in accordance with the protocol of the Institutional Animal Care and Use Committee, Yonsei University Wonju College of Medicine (Ethical approval no: YWC-191115-1).

2.2. Experimental Design

This experiment was designed as shown in Figure 1. TMT (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterilized 0.9% saline and single dose of 2.6 mg/kg injected ip into the mice except the NC group. For the PC group, lithium chloride (LC) (Sigma-Aldrich, St. Louis, MO, USA) (50 mg/kg body weight) was administered twice by intraperitoneal(IP) injection at 0 and 24 h after TMT injection.Previous conducted studies have shown LC as a potent neuroprotective agent and was found to ameliorate neurodegeneration, neuroinflammation, neurotoxicity and behavioral disability in TMT-induced hippocampal neurodegeneration model [19, 21]. For the treatment of H_2 group, 2% H_2 gas was produced from H_2 generating device designed and provided by the company (GOOTZ Co., Ltd., Suwon, Gyeonggi-do, Korea). The mice were administered 2% H_2 gas for 30 min once a day for 4 wks. To investigate the effect of H_2 inhalation, seizure behaviors were observed for 4 consecutive days after TMT treatment. Furthermore, to assess the TMT-induced memory deficits, hippocampus-dependent memory tests (Y-maze memory test) were performed at 7^{th} day of TMT induction, at which time the seizure behavior had disappeared. Body weight was measured once a week for 4 wks. At the end of the experiment, the mice were sacrificed using isoflurane anesthetics (Hana Pharm. Co., Hwaseong, Korea). After sacrificing the

mice, their blood samples were collected from retro-orbital veins in the tubes and serum was separated by centrifugation at 14,000 rpm for 5 min at 4℃. To obtain brain lysate samples, the heads of the mice were decapitated and the hippocampal region of the brain was separated and homogenized in ice-cold Radioimmunoprecipitation assay (RIPA) lysis buffer (Pierce Biotechnology Inc., IL, USA) with a protease inhibitor cocktail (Sigma Chemical Co., St Louis, USA) using bead milling method (QIAGEN Tissue Lyser II, manufactured by Retsch, Goleta, CA, USA) at 30 frequency/s for 10 min. Thereafter, the brain lysate homogenate was centrifuged at 14,000 rpm for 10 min at 4℃, and the protein concentration of the obtained supernatant was checked using a bicinchoninic (BCA) protein assay kit (Takara Bio, Shiga, Japan) and was normalized. Both collected serum and brain lysates samples were stored at −80°C until further use. Furthermore, ROS, NO, Ca^{2+}, malondialdehyde(MDA), glutathione peroxidase assay(GPx) and catalase activities were measured as OS-related markers. G-CSF, IL-1β, TNF-α, and vascular endothelial growth factor(VEGF), were measured as inflammation-related markers, and Bcl-2, Bcl-2 associated X protein(Bax) as apoptotic markers. In addition, apolipoprotein E(Apo-E), Aβ-40 and phospho-tau (p-tau) were measured as AD-related markers.

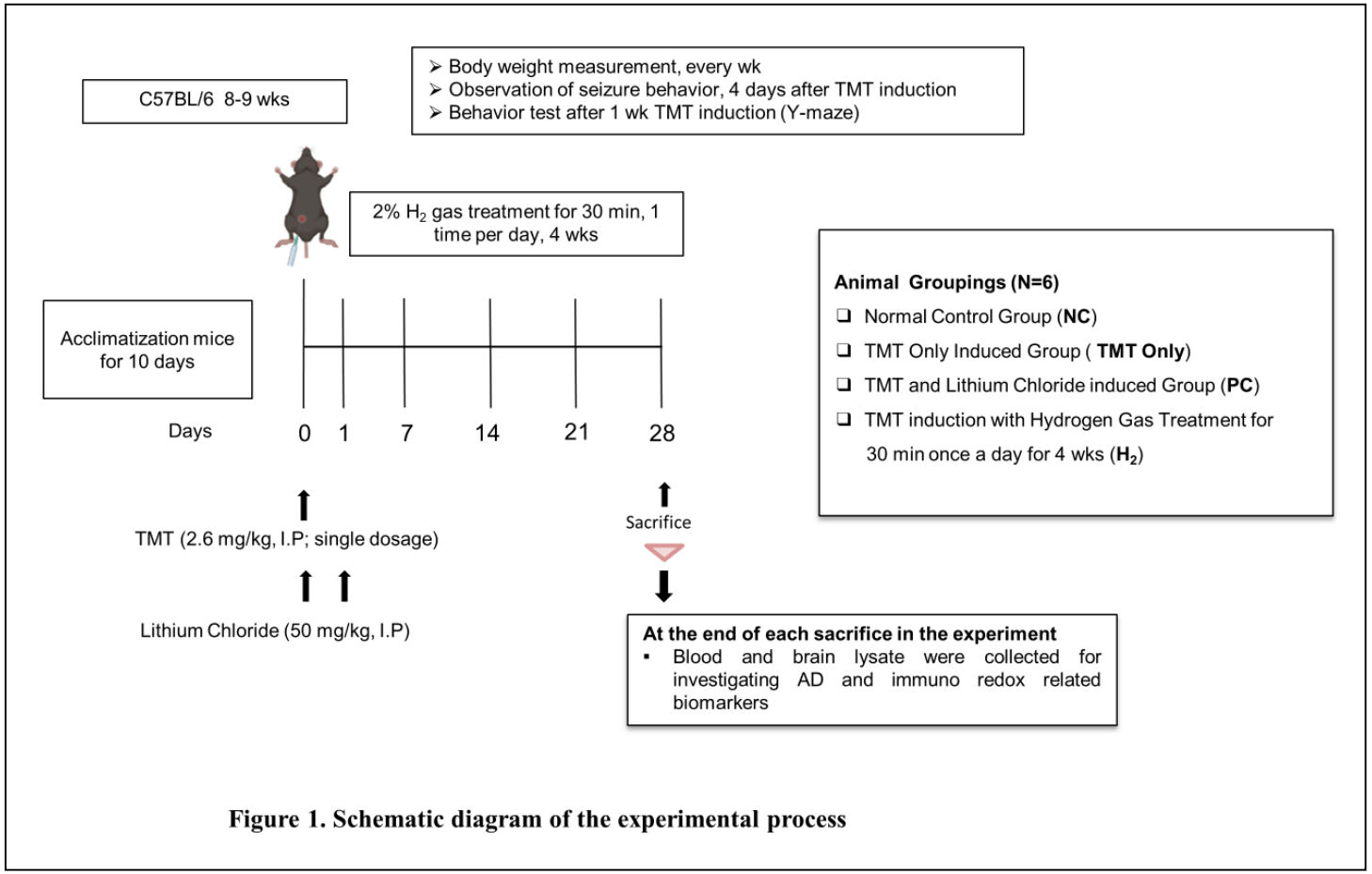

Figure 1. Schematic diagram of the experimental process

2.3. Seizure Scoring and Body Weight Measurement

Seizure and tremor behavior tests were performed in mice until 4 days after TMT induction. Scores were assigned for behavioral changes in mice, ranging between 1 (aggression), 2 (weak tremor), 3 (systemic tremor), 4 (tremor and spasmodic gait), and 5 (death) [31]. In addition, body weight measurements were conducted every week for 4 wks to obtain the baseline data of all experimental mice.

2.4. Y-Maze for Behavioral Test

After 7 days of TMT injection, a Y-maze behavioral test was performed in mice according to their grouping. The maze was made from black Y-shaped plastic, and the arms were at an angle of 120° from each other. Each mouse was allowed to freely move in the maze for 8 min, and the sequence of arm entries was recorded with a Smart 3.0 video tracking system (Panlab, Harvard, USA). The spatial cognition (%) ability was calculated as follows: actual alternation/ (total number of arm entries − 2) × 100. In addition, the total distance entered in the zone was determined.

2.5. Detection of ROS Level

ROS levels were measured using dichloro-dihydro-fluorescein diacetate (DCFH-DA) reagent (Sigma, St. Louis, MO, USA) following the manufacturer's instructions. Briefly, 10μL of serum and brain lysates samples and 100 μL of 10 μM DCFH-DA were mixed in a 96-well black plate and incubated for 30 min at 37℃. The fluorescence was measured using a DTX multi-mode microplate reader (Beckman Coulter Inc., Brea, CA, USA) at 488 nm excitation/525 nm emission.

2.6. Detection of NO Level

NO levels were measured using the Griess reagent (iNtRON Biotechnology, Sungnam, South Korea) following the manufacturer's instructions. Briefly, 50μL of serum and brain lysates samples were mixed with an equal volume of Griess reagent in a 96-well microplate and incubated at room temperature for 15 min. The absorbance was measured at 540 nm using SpectraMax® *ABS* Plus (Molecular Devices, San Jose, CA, USA). The NO_2^- concentration was calculated by a standard curve graph which was generated by serial two-fold dilutions of sodium nitrate.

2.7. Analysis of Antioxidant Enzyme Activities

Intracellular levels of endogenous antioxidant enzymes (catalase and GPx) were measured using the BioVision kit (BioVision, Inc., Milpitas, CA, USA) following the manufacturer's instructions. Briefly, 78 µL of samples (10 µL from stock and 68µL assay buffer) for the catalase assay and 50 µL of samples (10 µL from stock and 40µL assay buffer) for the GPx assay were added into a 96-well microplate, and the plates were incubated for 30 min. The optical densities of catalase (570 nm) and GPx (340 nm) were measured using SpectraMax® *ABS* Plus (Molecular Devices, San Jose, CA, USA). The results of catalase and GPx activities were expressed in nmol/min/mL and mU/mL.

2.8. Detection of Ca^{2+} Activity

Intracellular Ca^{2+} activities were measured in serum and brain lysates using a Ca^{2+} colorimetric assay kit (BioVision, CA, USA) following the manufacturer's instructions. In brief, 50 µL of each sample was added to a 96-well microplate, and the plate was incubated for 30 min. The optical density was measured at 590 nm using SpectraMax® *ABS* Plus (Molecular Devices, San Jose, CA, USA). The results were expressed in mM.

2.9. Detection of MDA Activity

The level of MDA in serum and brain lysates was measured using a thiobarbituric acid reactive substances assay kit (BioVision, Milpitas, CA, USA). The assay was performed according to the manufacturer's instructions. The reaction product was measured calorimetrically at 532 nm using the SpectraMax® ABS Plus microplate reader (Molecular Devices, San Jose, CA, USA).

2.10. Detection of Inflammatory Cytokines and Vascular Endothelial Growth Factor by Multiplex Assay

Cytokine and chemokines profiling was performed using the Milliplex® MAP Mouse Cytokine/Chemokine Magnetic Bead Panel 96-well plate assay (Millipore Corporation, Billerica, MA, USA) as a luminex-based multiplex technology. Granulocyte colony-stimulating factors (G-CSF), IL-1β, TNF-α, and VEGF were measured by using a multiplex immunoassay following the manufacturer's protocol. In brief, each standard concentration was resuspended in standard diluents, and serial dilutions of the standard were prepared. The bead mixture was added to the standard and serum and brain lysates. The plate was incubated overnight (18 h) at 4 ℃ and was proceeded by a washing step. Detection antibody was added, and the plate was incubated at room temperature for 1 h. Streptavidin-phycoerythrin mix

was added and the plate was incubated at room temperature for 30 min. After the washing step, an assay buffer was added, and the plate was analyzed using the Luminex 200 Bio-Plex instrument.

2.11. Detection of Total Apo-E by ELISA

Apo-E levels in serum and brain lysates were detected using the Rat Apo-E ELISA kit (MyBioSource, Inc. San Diego, CA, USA), and analyses were performed according to the manufacturer's instructions. The concentration of Apo-E was visualized at 450 nm using a SpectraMax® ABS Plus spectrophotometer (Molecular Devices, San Jose, CA, USA), and the concentration of Apo-E was calculated using the standard curve.

2.12. Western Blot Analysis

The hippocampi of C57BL/6 mice were homogenized by using bead milling method (QIAGEN Tissue Lyser II, manufactured by Retsch, USA) at 30 frequency/sec for 10 min, incubated with 500μL lysis RIPA buffer at 4℃, and centrifuged at 14,000 rpm for 10 min. The liquid supernatant was collected and BCA protein assay kit (Takara, Shiga, Japan) was used to measure the total protein

concentration. Equal amounts of protein samples (20 μg) were separated on 12% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Pall., Ann Arbor, MI, USA) at 300 mA for 2 h. After transferring, the membranes were blocked with a blocking buffer (Takara, Shiga, Japan) for 1 h at room temperature. The membranes were further incubated with the primary antibodies Beta-actin (Cell Signaling Technology, Massachusetts, USA), Aβ-40 (My Biosource, San Diego, CA, USA), p-tau-(Ser404) (Cell Signaling Technology, Danvers, MA, USA), Bcl-2 and Bax (Cell Signaling Technology, Danvers, MA, USA) in the dilution 1:2000 at 4℃ overnight. After washing thrice with 1×Tris Buffered Saline and Tween (1×TBST), the membranes were treated with horseradish peroxidase-(HRP) conjugated anti-rabbit secondary antibody (dilution 1:5000; Cell Signaling Technology) in 1×TBST for 2 h at room temperature. After washing thrice, the bound antibodies were detected by an enhanced chemiluminescence kit (ECL Pierce Biotechnology, ThermoFisher Scientific, USA), using the UVP Bio Spectrum 600 Imaging System (UVP, LLC, Upland, CA, USA). Band intensity was analyzed using ImageJ software (Version 150-win Java, Bethesda, MD, USA).

2.13. Data Management and Statistical Analysis

Values are represented as mean ± standard error of the mean (SEM). All data were analyzed and compared by two-way analysis of variance (ANOVA), followed by a multiple comparison test using Graph Pad Prism 8.0 software package (Graph Pad, La Jolla, CA, USA). Differences were considered to be statistically significant at $p < 0.05$.

III. RESULTS

3.1. Effects of H_2 Gas Inhalation in the TMT-Induced C57BL/6 Mice on Seizure Behavior and Body Weight Measurement

TMT induction is known to induce seizure behavior in mice [13]. In our study, we tested the effects of H_2 gas inhalation on seizure-like behaviors in TMT-induced mice. During the experiment, with TMT induction, symptoms such as tremors, seizures, and aggressive behavior were observed in mice for 4 days. However, administration of PC and H_2 treatment resulted in significant decrease in seizure-like behaviors from day 2 to 4 (Figure 2A). In day 2 ($p < 0.05$), day 3 ($p < 0.001$) and day 4 ($p < 0.01$) behavioral seizure score showed significant reduction in PC-treated group as compared to TMT Only group. Likewise, administration of H_2 also significantly decreased seizure-like behaviors in TMT-induced mice from day 2 ($p < 0.05$), day 3 ($p < 0.05$) and day 4 ($p < 0.001$) as compared to TMT Only group (Figure 2A). These results indicate that administration of H_2 gas inhalation reduces seizure-like behaviors in TMT-induced mice. Additionally, the body weight was measured once a week for 4 wks to obtain baseline data. Our results showed that TMT induction led to a slight decrease in body weight on the 7^{th} day in the TMT Only group, compared to the NC, PC, and H_2 groups. However, the difference was not found significant (Figure 2B).

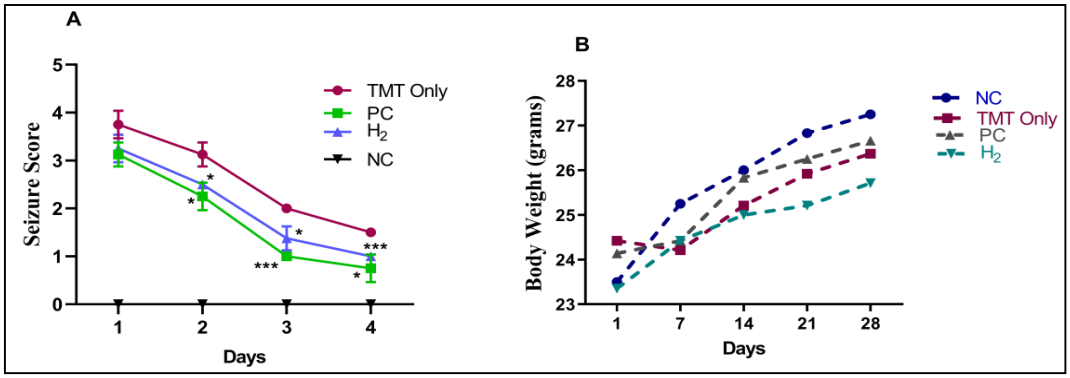

Figure 2. Effects of H_2 gas inhalation treatment on the seizure behavior (A) and body weight (B) in the TMT-induced C57BL/6 mice. Data are shown as mean ± SEM for 4 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, ***$p < 0.001$ compared with TMT Only group

3.2. Effects of H_2 Gas Inhalation on TMT-induced Cognitive Dysfunction

To investigate the effects of H_2 gas inhalation on TMT-induced cognitive dysfunction, TMT was injected ip in mice, and 2% H_2 gas was administered for 4 wks. Since the Y-maze test was conducted to determine spatial cognition and short-term abilities, we performed the Y-maze test on day 7 after TMT induction to determine the willingness of mice to explore new circumstances and to assess their short-term memory. We found that spontaneous alternation (%) of the TMT Only group was decreased compared to the NC, PC, and H_2 groups (Figure 3A). In addition, we found significantly increased distance travel in the maze compartment zone in the TMT Only group ($p < 0.001$) compared to those of the NC, PC, and H_2 groups (Figure 3B). With this, one of the previous studies has reported that TMT-exposed animals showed cognitive impairment as well as hyperactive behavior as compared to control group [13], which is consistent with our findings in this study. Therefore, our results suggest that H_2 could attenuate the TMT-induced impairment of spatial working memory and hyperactivity in mice. However, more types of maze experiments are needed to fully confirm this notion.

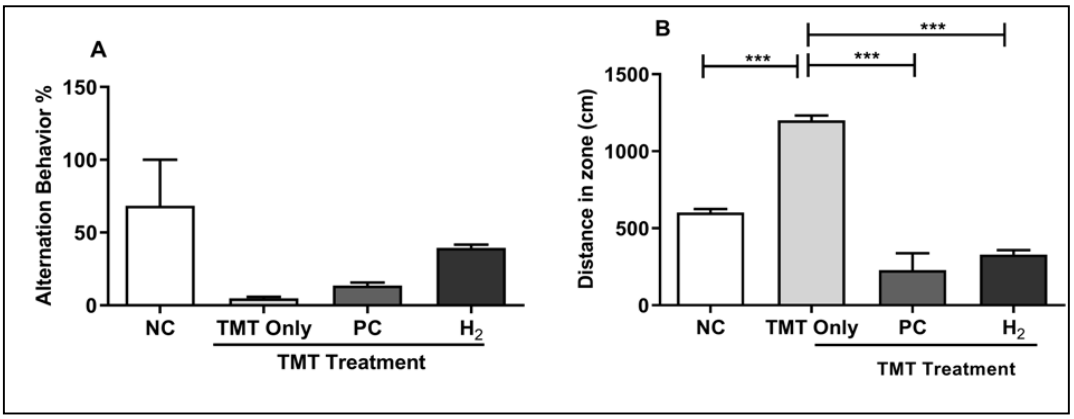

Figure 3. Effects of H_2 gas inhalation treatment on TMT-induced spatial cognitive impairment in C57BL/6 mice. After TMT (2.6mg/kg body weight) was administered IP to mice (n=6), 2% H_2 gas (H_2group) and lithium chloride (50 mg/kg body weight, IP) (PC group) were treated. **(A)** Alternation behavior on day 7 **(B)** Distance travel in Y-maze compartment. Data are shown as mean ± SEM for 4 mice. Significant difference was analyzed with ANOVA Tukey's test. ***$p < 0.001$

3.3. Effects of H_2 Gas Inhalation on TMT- induced ROS and NO Levels in Mice Serum and Brain

OS is considered as one of the known toxic mechanisms of TMT induction [13]. Thus, to investigate the effects of H_2 gas inhalation on TMT-induced neuronal toxicity, we analyzed the total ROS and NO levels in both the serum and brain lysates of C57BL/6 mice. Our results showed a significant increase in total intracellular ROS levels ($p < 0.05$) and NO levels ($p < 0.01$) in the serum of the TMT Only group compared to the NC group (Figure 4A, C). Additionally, we found that ROS and NO levels were markedly increased in the brain lysates of the TMT Only group ($p < 0.001$, and $p < 0.01$) compared to the NC group (Figure 4B, D). In contrast, ROS levels were significantly decreased in the brain of the H_2-treated ($p < 0.01$) and PC-treated groups ($p < 0.01$), compared to that of the TMT Only group (Figure 4B). Additionally, upon H_2 treatment, TMT-induced NO levels were found to be significantly decreased ($p < 0.01$) compared to the TMT Only group (Figure 4D). Taken together, our results showed that with H_2 gas treatment, both the OS markers, i.e., ROS and NO, were effective in repairing TMT-induced neuronal damage.

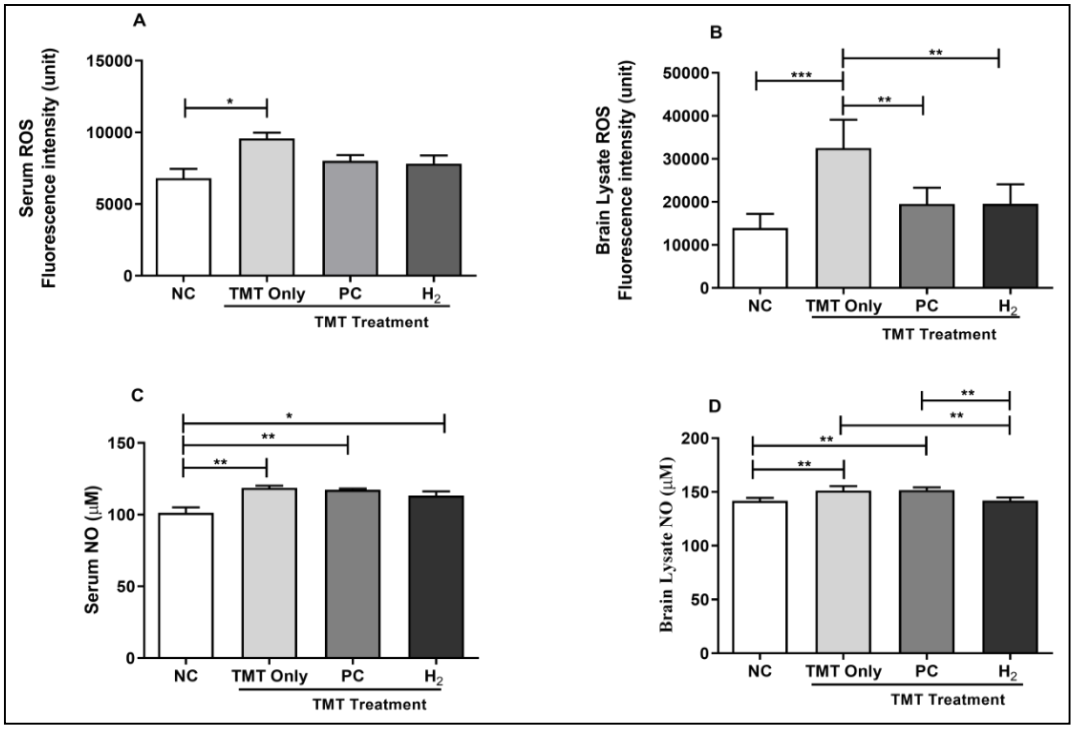

Figure 4. Effects of H_2 gas inhalation treatment on total ROS and NO levels in TMT-induced C57BL/6 mice after 4 wks. (A) Serum ROS level, (B) Brain lysates ROSlevel, (C) Serum NO level, (D) Brain lysates NO level. Data are shown as mean ± SEM for 4 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.4. Effects of H_2 Gas Inhalation on TMT- induced Ca^{2+} and MDA Levels in Mice Serum and Brain

The role of lipid peroxidation and intracellular Ca^{2+}has been extensively studied in AD pathogenesis [11, 16]. MDA is an indicator of lipid peroxidation induced by OS [9]. In our study, we investigated the effects of H_2 inhalation on TMT-induced Ca^{2+} and lipid peroxidation levels in C57BL/6 mice in both the serum and brain lysates. As shown in Figure 5A and 5B, Ca^{2+} level in both serum and brain lysates of the TMT Only mice showed higher values as compared to PC and H_2 groups. However, with LC and H_2 treatment the Ca^{2+} level was found to be decreased in both groups as compared to TMT Only group but without significant difference in both serum and brain lysates. Furthermore, we evaluated the effects of H_2 inhalation on the TMT-induced MDA level. As shown in Figure 5C, the MDA level in TMT Only ($p < 0.01$), PC ($p < 0.05$), and H_2 ($p < 0.01$) groups was significantly increased in the serum of TMT-injected mice compared with those of the NC mice. In contrast, we found significant increased MDA level in TMT Only group in brain lysates compared with those of the NC mice. However, with PC and H_2 administration the level of MDA was significantly decreased in PC ($p < 0.01$) and H_2 group ($p < 0.001$) with those of TMT Only group (Figure 5D). These results demonstrated that TMT effectively induces damage in the brain by increasing intracellular Ca^{2+} and MDA

levels, while H_2 treatment effectively repairs the damage caused by TMT and might have therapeutic effects.

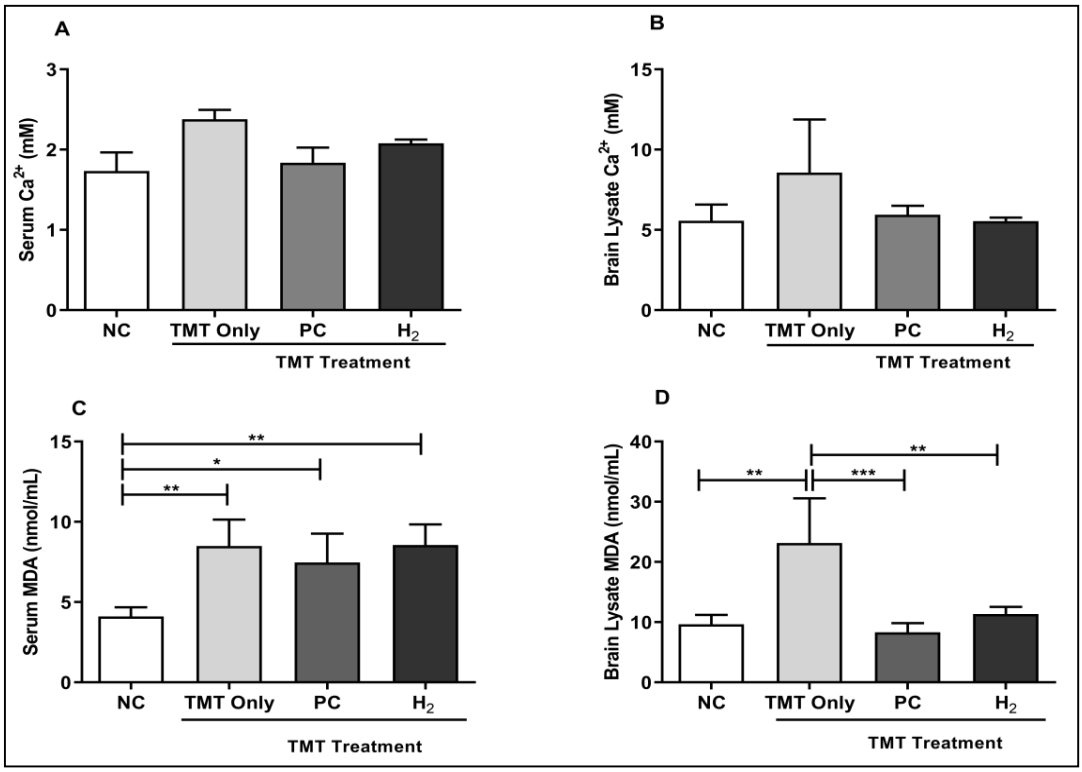

Figure 5. Effects of H_2 gas inhalation treatment on Ca^{2+} and malondialdehyde (MDA) levels in TMT-induced C57BL/6 mice after 4 wks. (A) Serum Ca^{2+} level, (B) Brain lysates Ca^{2+} level, (C) Serum MDA level, (D) Brain lysates MDA level. Data are shown as mean ± SEM for4 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.5. Effects of H_2 Gas Inhalation on Antioxidative Enzyme Activities in TMT-induced Damage in Mice Serum and Brain

To investigate the effects of H_2 gas inhalation on OS imbalance in TMT-induced damage, we measured the activities of antioxidative enzymes, such as catalase and GPx in serum and brain lysates. Our results showed that catalase activity in serum was significantly decreased in NC ($p < 0.05$) and H_2 groups ($p < 0.05$) compared with those of the TMT Only group (Figure 6A). In addition, our brain lysates result showed significant decreased catalase level in H_2-treated group ($p < 0.01$) compared with those of the TMT Only group (Figure 6B). Moreover, we investigated the GPx level in both serum and brain lysates. Our results showed significant decreased GPx activity in both NC ($p < 0.01$) and H_2 ($p < 0.05$)groups in serum (Figure 6C). Likewise, we found significant decreased GPx activity in NC ($p < 0.05$), PC ($p < 0.05$) and H_2 ($p < 0.05$) groupscompared with those of the TMT Only group in brain lysates (Figure 6D). These results demonstrated that elevated catalase and GPx levels in the serum and brain with TMT induction may reflect a compensatory rise in antioxidant response to oxidative damage.

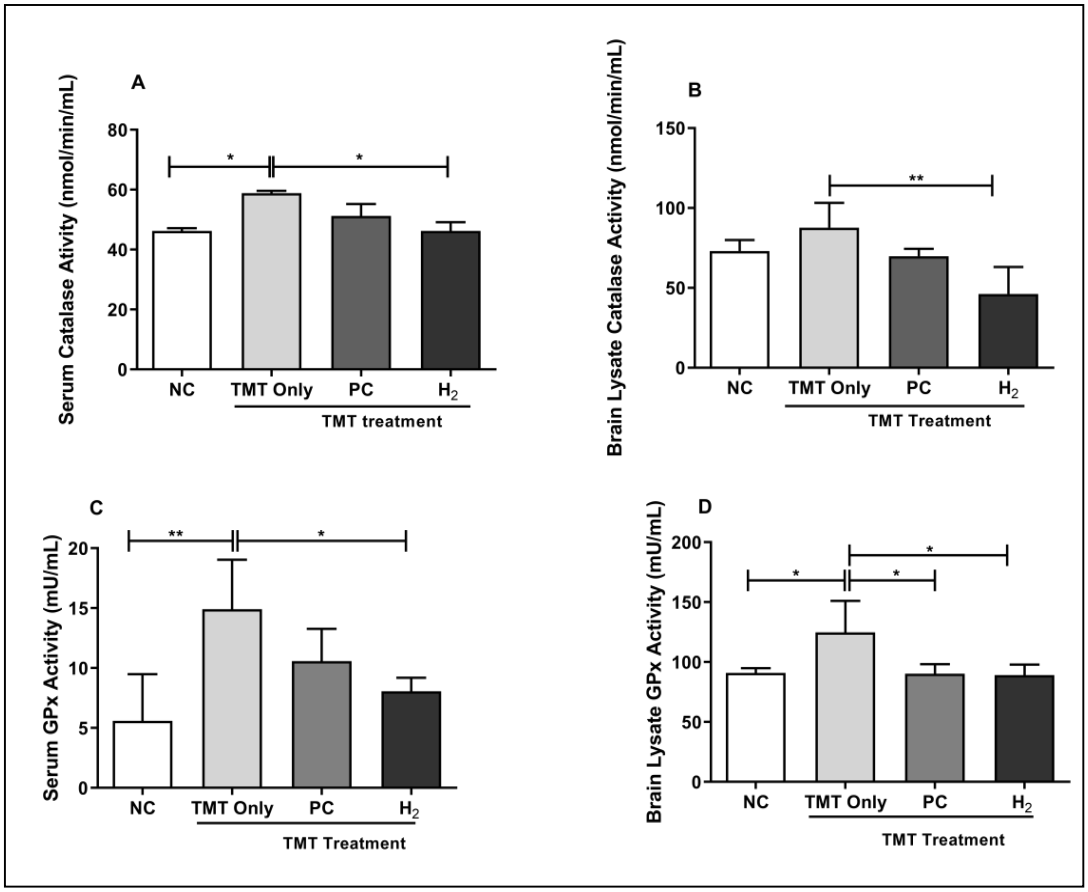

Figure 6. Effects of H_2 gas inhalation treatment on total intracellular catalase and GPx levels in TMT-induced C57BL/6 mice after 4 wks. (A) Serum catalase level, (B) Brain lysates catalase level, (C) Serum GPx level, (D) Brain lysates GPx level. Data are shown as mean ± SEM for 4 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, **$p < 0.01$

3.6. Effects of H_2 Gas Inhalation on Inflammatory Cytokines and Vascular Endothelial Growth Factor in TMT-Induced Damage in Mice Serum and Brain

It is well established that TMT-induction plays a role in neuroinflammation [13, 23]. Therefore, we investigated the expression of inflammatory cytokines such as G-CSF and TNF-α and vascular endothelial growth factor (VEGF) in the serum and brain lysates. Our results showed that the administration of TMT significantly decreased the granulocyte colony-stimulating factors (G-CSF) level in serum compared with those of the NC ($p < 0.01$), PC ($p < 0.05$) and H_2 ($p < 0.05$) groups (Figure 7A). In addition, TNF-α level were found significantly increased in TMT Only group, however, with PC and H_2 treatment the level of TNF-α (inflammatory cytokines) were significantly decreased in PC ($p < 0.01$) and H_2group ($p < 0.01$) in serum (Figure 7B). Moreover, we measured the VEGF level in serum of mice. Our results showed that the level of VEGF was significantly decreased in the TMT Only group ($p < 0.01$), PC ($p< 0.05$), and H_2 ($p < 0.05$) groupscompared with those of the NC group (Figure 7C).

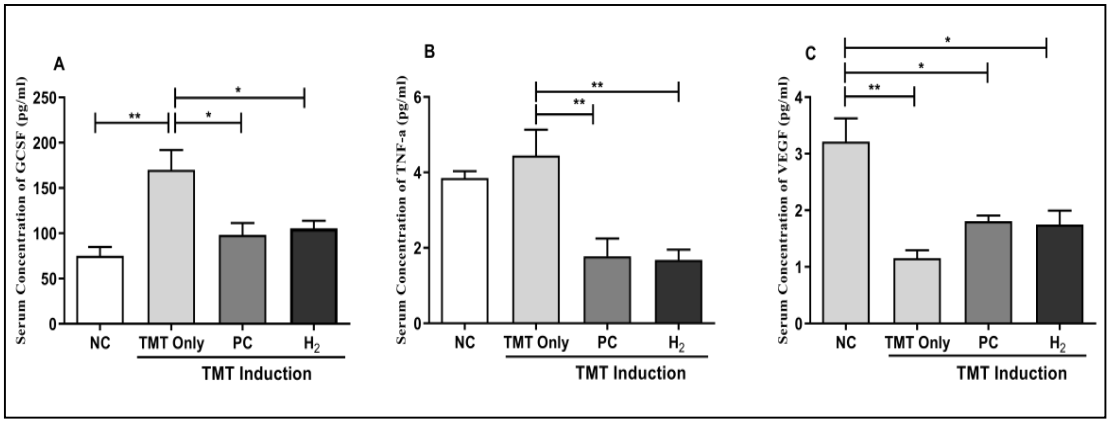

Figure 7. Effects of H_2 gas inhalation treatment on inflammatory cytokines and VEGF in serum in TMT-induced C57BL/6 mice after 4 wks. (A) G-CSF, (B) TNF-α, (C) VEGF. Data are shown as mean ± SEM for 4 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, **$p < 0.01$

Furthermore, our brain lysates results showed significant reduction of G-CSF level in PC ($p < 0.05$) and H_2($p < 0.05$) groups compared with that of the TMT Only group (Figure 8A). In addition, level of IL-1β cytokine was found markedly increased in the TMT Only group and decreased in the NC, PC, and H_2groups but without significant difference (Figure 8B). Moreover, TNF-α level were found significantly increased in TMT Only group, however, with PC and H_2 treatment the level of TNF-α were significantly decreased in H_2 group ($p < 0.01$) in brain lysates (Figure 8C). Additionally, VEGF expression was significantly increased in the H_2-treated group as compared to those of TMT Only group (Figure 8D). Collectively, these results indicate that H_2 effectively attenuates TMT-induced neurotoxicity and cognitive damage by inhibiting inflammatory cytokines and upregulating VEGF.

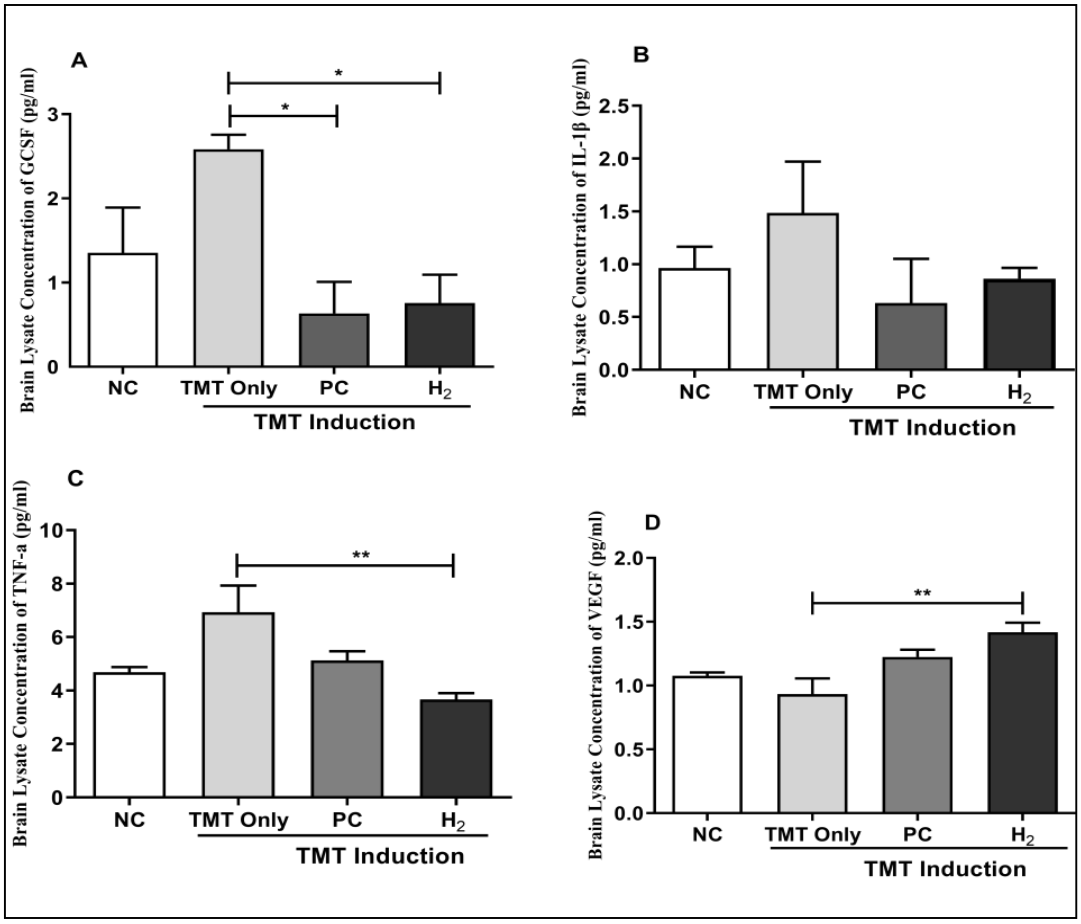

Figure 8. Effects of H_2 gas inhalation treatment on inflammatory cytokines and VEGF level in brain lysates in TMT-induced C57BL/6 mice after 4 wks. (A) G-CSF, (B) IL-1β, (C)TNF-α, (D) VEGF. Data are shown as mean ± SEM for 4 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, **$p < 0.01$

3.7. Effects of H_2 Gas Inhalation on Apo-E Activities in TMT-Induced Damage in Mice Serum and Brain

The Apo-E gene is strongly associated with the risk of AD [37]. To verify the role of TMT- induction in Apo-E activity, we further evaluated total Apo-E levels in blood and brain lysates using ELISA. We found that Apo-E levels were significantly increased in the TMT Only group ($p < 0.05$) in serum compared to the NC group, whereas in the brain lysate, Apo-E activity was significantly decreased in PC ($p < 0.001$) and H_2 ($p < 0.001$) groups compared with those of the TMT Only group (Figure 9A, B). Taken together, our results showed that TMT induction of Apo-E levels effectively induced brain damage and was associated with AD progression, whereas H_2 treatment effectively improved brain damage associated with progression of TMT-induced AD.

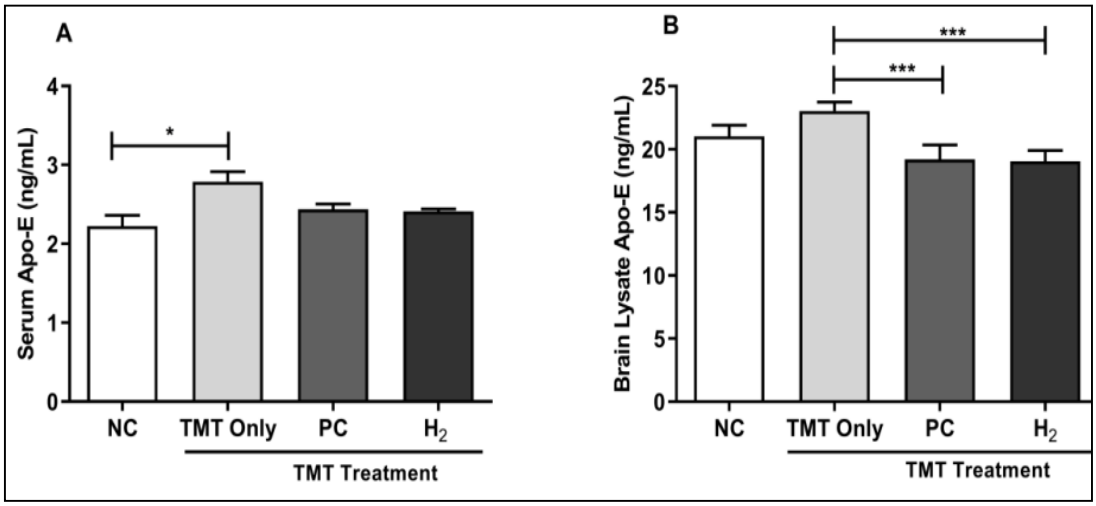

Figure 9. Effects of H_2 gas inhalation treatment on intracellular Apo-E activities in TMT-induced C57BL/6 mice after 4 wks. (A) Serum Apo-E level, (B) Brain lysate Apo-E level. Data are shown as mean ± SEM for4 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, ***$p < 0.001$

3.8. Effects of H_2 Gas Inhalation on AD-related Biomarkers in Brain Tissue in TMT-induced Damage

We verified the underlying molecular mechanisms of H_2 gas inhalation against TMT-induced neurotoxicity and cognitive impairment and evaluated the protein expression of AD-related biomarkers such as Aβ-40 and phospho-tau (p-tau)-Ser404 by western blot analysis. In our results, we observed various sizes of bands of Aβ oligomers/aggregates in mouse brain lysates. In addition, we observed that a wide range of Aβ molecules such as oligomers and aggregates with upward molecular size was intensified in TMT-induced group as compared to NC group indicating that AD was induced in the hippocampus following TMT induction. However, with LC and H_2 treatment, the expressions of Aβ-40 oligomers/ aggregates were found to be decreased as compared to TMT Only group (Figure 10A). Similarly, we found the expression levels of p-tau-(Ser404) was significantly increased in the TMT Only group *(p <0.001)* compared to the NC group, however, with PC *(p < 0.05)* and H_2 treatment *(p < 0.001)*, the expression of p-tau-(Ser404) was significantly decreased (Figure 10B).

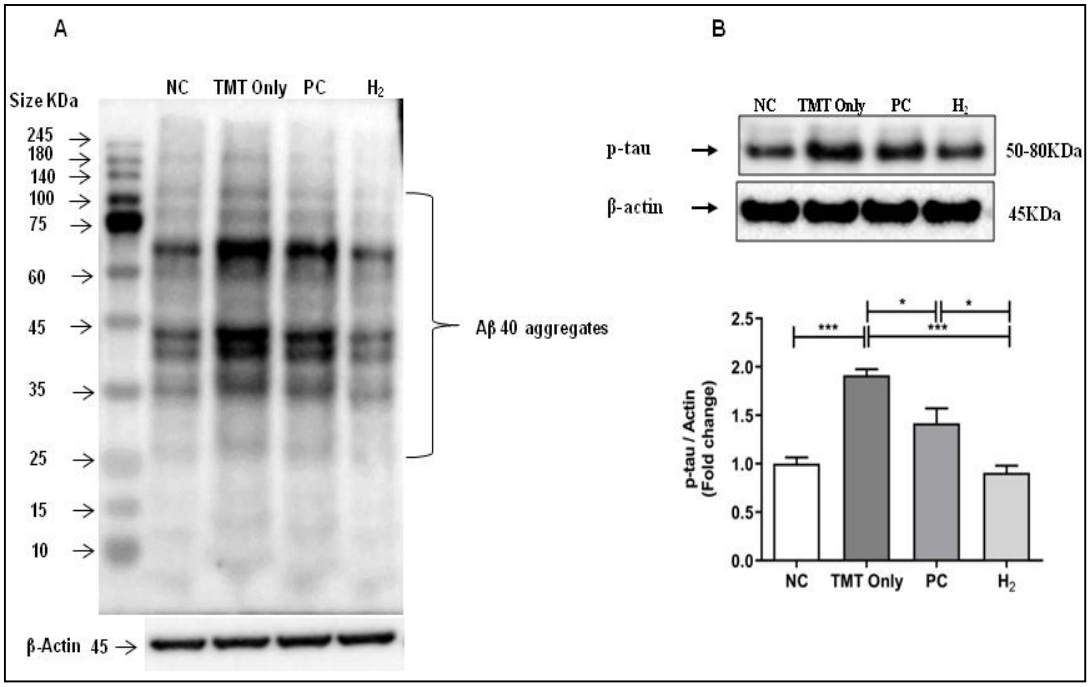

Figure 10. Effects of H_2 gas inhalation treatment on the Aβ-40 (mouse monoclonal-32A1) and p-tau-(Ser404) protein levels in brain lysates in TMT-induced C57BL/6 mice after 4 wks. (A) The antibody-reactive bands of mouse Aβ-40 in the brain lysates, (B) The antibody-reactive band of p-tau-(Ser404) protein levels in brain lysates. The bar graphs represent the band intensity of each protein marker normalized to total. The data are represented as mean ± SD for 3 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.9. Effects of H_2 Gas Inhalation on Bcl-2 and Bax in Brain Tissue in TMT-Induced Damage

We investigated underlying molecular mechanisms of H_2 gas inhalation against TMT-induced neurotoxicity and evaluated the protein expression of Bcl-2 and Bax protein by western blot analysis. We found that the expression level of Bcl-2 were significantly decreased in the TMT Only group ($p < 0.001$) compared to the NC group. However, with LC ($p < 0.01$) and H_2 ($p < 0.001$)treatment, the expression of Bcl-2 was significantly increased (Figure 11A). In contrast, we found that the expression levels of Bax were significantly increased in the TMT Only group ($p < 0.001$) compared to the NC group, however, with LC ($p < 0.01$) and H_2 ($p < 0.001$) treatment the expression of Bax were significantly decreased as compared to TMT Only group(Figure 11B), indicating that TMT-induced apoptosis was attenuted by H_2 gas inhalation treatment in the brain.

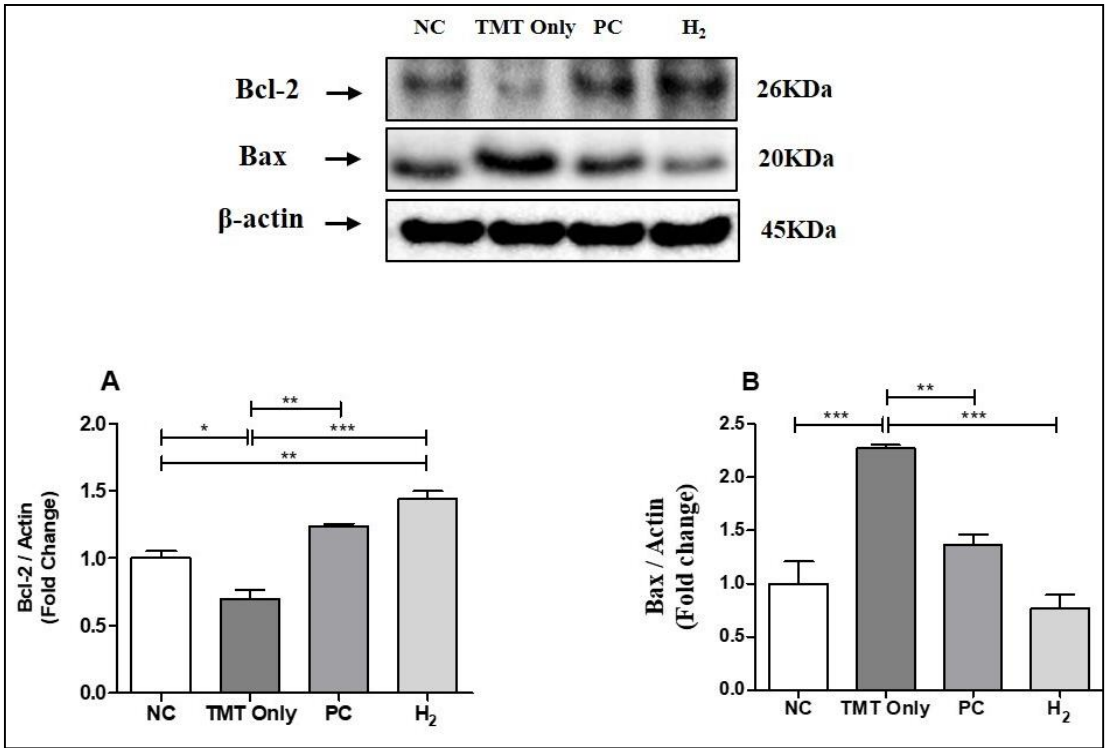

Figure 11. Effects of H_2 gas inhalation treatment on the Bcl-2 and Bax protein levels in brain lysates in TMT-induced C57BL/6 mice after 4 wks. (A) The antibody-reactive quantified band intensity of Bcl-2 in the brain lysates, (B) The antibody-reactive quantified band intensity of Bax protein in brain lysates. The bar graphs represent the band intensity of each protein marker normalized to total. The data are represented as mean ± SD for 3 mice. Significant difference was analyzed with ANOVA Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

IV. DISCUSSION

The present study investigated the therapeutic effects of H_2 gas inhalation on TMT-induced neurotoxicity and cognitive impairment in mice. Our findings indicate that H_2 gas treatment ameliorates TMT-induced OS, and inflammatory and AD-related markers in C57BL/6 mice. Several studies have reported that neurodegenerative diseases are characterized by progressive neuronal death, resulting in the loss of neurons in specific areas, such as the hippocampal region of the brain, and causes spatial cognitive impairment such as AD [2, 3, 38]. Recently, researches in H_2 field have been emerging due to its great biosafety and effectiveness as a medical gas [39, 40]. Studies have shown that H_2 is highly capable to penetrate the biological membranes including the BBB. Thus, these characteristics of H_2 provides unrestricted access to the CNS, causing therapeutic effects in many CNS diseases, including ischemia stroke, intracranial hemorrhage, and neurodegenerative diseases [41- 44]. Moreover, previous conducted studies have shown that administration of H_2 inhalation attenuated redox stress, inflammatory and OS mediators and prevented from BBB disruption by reducing the activation of mast cells and degranulation which result in amelioration of neurological condition [24, 39, 41]. In addition, studies have shown that inhalation of H_2 gas (1-4%) was found effective for the improvement of acute and chronic

neurological conditions in different clinical and animal models [31, 39]. Additionally, one of the recent published studies has confirmed about safety and effectiveness of administration of 3% H_2 on acute cerebral infarction patients [31]. Moreover, administration of 2% H_2 gas inhalation has shown the alleviated BBB impairment and memory dysfunction in brain through regulation of antioxidative pathway in mice [45]. Therefore, the attenuation of neuronal death through H_2 treatment might be a novel strategy for the prevention and treatment of cognitive impairments like AD.

TMT is a commonly used neurotoxin for constructing *in vivo* models of neurodegenerative diseases like AD, since TMT injection can specifically alter conditions such as tremors, seizures, and spatial recognition memory, which are associated with hippocampal dysfunction in the brain. Animals exposed to TMT experience spontaneous seizures and elevated neuronal excitability [13, 15, 16]. Interestingly, studies have reported that H_2 has potential to protect against many neurological diseases, including cerebral injury, neuropathic pain, cognitive impairment and AD [32, 46-48]. Our results showed that TMT induction induces aggressive behavior, seizures, whole body tremors, and memory deficits in mice. Moreover, reduction in seizure behavior and improved memory were observed following the repeated H_2 gas inhalation in mice. Therefore, these results suggest that repeated administration of H_2 gas attenuates TMT-induced toxicity in the

hippocampal region of the brain. However, detailed further study investigations are required to verify this claim.

Compelling evidences suggest that extracellular Aβ plaques and hyperphosphorylated tau protein are the key pathological hallmarks in AD [4-6]. The deposition and aggregation of Aβ plaques and hyperphosphorylated tau proteins leads to excessive production of ROS and RNS which causes OS, chronic neuroinflammation, and dysfunction in mitochondrial function in the brain, altogether causes neuronal loss and protein misfolding [49, 50]. In TMT-induced neurotoxicity and damage, OS is considered as one of the main mechanisms underlying neurodegeneration. Previous evidences have shown that TMT induces ROS, RNS, and MDA (lipid peroxidation marker) both *in vivo* and *in vitro* [51-53]. These oxidative conditions could affect behavioral abnormalities and imbalance of the antioxidant defense system, and consequently cause neuronal cell death in TMT-induced animals [51, 52]. In addition, endogenous compensatory anti-oxidative responses are activated simultaneously in the brain for the recovery of various neurological damage conditions, such as TMT induction. A previous conducted study has shown that H_2 selectively reduces highly toxic ROS such as hydroxyl radical and peroxynitrite and protects the cells against OS [24]. In addition, another study has shown that administration of H_2 decreases OS related markers such as hydroxyl-2-nonenal, NO and MDA in patients and rodents [54]. In our study,

we observed a significant increase in intracellular ROS, NO, and MDA levels in the TMT-induced mice, whereas H_2 gas treatment significantly attenuated these oxidative markers in both the brain and blood. In contrast, our results showed that the activities of antioxidant enzymes such as catalase and GPx were increased in the TMT-induced group and decreased in the PC and H_2 groups. This might be due to the compensatory mechanism of the antioxidant defense system against TMT-induced damage. Moreover, previous publications on hydrogen effects have reported that the administration of H_2 alleviates neurotoxicity by decreasing levels of OS biomarkers [3, 25]. Several studies have reported a connection between Ca^{2+} homeostasis disruption and the development of neuro-diseases like AD [55, 56]. Moreover, at the cellular level, it has been reported that abnormal amyloid accumulation induces an upregulation of neuronal Ca^{2+} signaling. Excessive Ca^{2+} release from the endoplasmic reticulum plays an important role in orchestrating the dynamics of the neuropathology of AD and associated memory loss and cognitive dysfunction [55, 56]. Additionally, one study reported that TMT-intoxicated injection dysregulated intracellular Ca^{2+} in hippocampal neurons [15]. Toward this, a previous study conducted on hydrogen administration demonstrated that exposure of cultured cells to the H_2-dependently autoxidized phospholipid species reduced Ca^{2+} signal and mediated the expression of different genes [35]. Our present results revealed that TMT-induction increased the values of intracellular Ca^{2+} activity in

both the serum and brain tissue. In addition, our findings show that H_2 gas administration reduced the values of the upregulated intercellular Ca^{2+} activity in the blood and brain. Taken together, these results indicate that administration of H_2 could effectively attenuate the OS markers both systematically and induced by TMT intoxication in mice brain, and aid recovery from TMT-induced neuronal damage.

Increasing evidences have shown that neuroinflammation plays a vital role in the progression of AD pathogenesis. Inflammation is not only known to be the key pathophysiological mechanism of Aβ plaques formation, but it is also a main cause of tau hyperphosphorylation, NFT formation and neurodegeneration [57, 58]. Chronically, neurotoxins such as Aβ can activate microglia to release cytotoxic factors, such as superoxide (O_2^-), NO, TNF-α, and IL- 1β in the brain that reliably trigger neuronal death [59, 60]. Another study reported that patients with AD had higher levels of pro-inflammatory cytokines, such as IL-4, IL-10, G-CSF, monocyte chemotactic protein 1, and TNF- α [61]. In contrast, VEGF signaling proteins have been reported to have protective effects against cognitive impairment [62]. Additionally, a study reported that TMT intoxication in mice induces neuronal death and the enhancement of inflammatory factors, including TNF- α and IL- 1β in the hippocampus [60]. Studies on H_2 have reported to exhibit anti-inflammatory actions in several studies [32, 54, 63]. Recently published study on H_2 also

demonstrated anti-inflammatory action in 3×Tg-AD mice [64]. In the present study, we examined cytokines such as G-CSF, IL- 1β, TNF- α, and VEGF in blood and brain tissues to investigate the effects of H_2 administration on TMT-induced cognitive damage. Our findings indicate that TMT intoxication in mice significantly increases the levels of inflammatory cytokines such as G-CSF and TNF-α in the blood, which was attenuated following 4 wks of H_2 gas administration. Additionally, H_2 treatment inhibited the expression of inflammatory cytokines, such as G-CSF, IL- 1β, and TNF- α in the brain tissue induced with TMT. Moreover, we found that H_2 treatment increased the VEGF protein levels in both blood and brain tissues in mice with TMT-induction. Thus, we may conclude that H_2 administration in TMT-induced mice exhibited repairing effects, which resulted in reduced TMT-induced neurotoxic damage by inhibiting inflammatory mediators, such as inflammatory cytokines, and improving cognitive function.

Furthermore, a previous study revealed that Apo-E protein is associated with a greater memory decline rate and cognitive dysfunction in patients with mild cognitive impairment [65]. Apo-E plays an important role in tau pathology, in the presence of Aβ, in the progression of AD and in the decline of cognitive function [66]. Additionally, it is known that accumulation and fibrillary deposition of the Aβ peptide in senile plaques and tau phosphorylation in neurofibrillary tangles are the major causes of cognitive dysfunction. In addition, toxic oligomers are generated

in brain by accumulation of Aβ which triggered OS-induced damage, neuroinflammation, hyperphosphorylation of tau, and dysregulation of metabolic pathways [4]. A study conducted by Park et al., reported that TMT induction increases the p-tau expression in TMT-induced mice [67]. Accordingly, previous conducted a study on H_2 showed amelioration of TMT-induced induced spatial learning and memory impairment in mice by regulation of Siah-1 [36]. In the present study, we evaluated the cognitive improvement effects of H_2 in TMT-induced mice by measuring the Apo-E levels in the blood and brain. Our results revealed a significant increase in Apo-E levels in both the blood and brain of TMT-induced mice. On the other hand, H_2 administration in mice for 4 wks after TMT-induction significantly decreased Apo-E levels in the brain. Additionally, we measured Aβ-40 and p-tau protein expressions using western blot in TMT-induced hippocampal brain samples. Our results showed that Aβ-40 and p-tau protein expressions were significantly increased in TMT Only group, whereas with H_2 and PC treatment, expressions of Aβ-40 and p-tau were significantly attenuated in TMT-induced mice.

It is well known that apoptosis is an important mechanism for TMT-induced hippocampal neuronal toxicity and damage [13, 16, 17]. Bcl-2 and Bax plays important role in promoting apoptosis whereas their roles are functionally opposite. Bcl-2 is known as the key cell apoptosis protein marker, whereas Bax acts to

promote apoptosis mechanism. In previous conducted study, administration of hydrogen-rich saline showed improved neurological function, and decreased neuronal apoptosis in rabbits by upregulation of Bcl-2 and downregulation of Bax protein in subarachnoid hemorrhage [68]. Therefore, to investigate the relationship between TMT-induction and H_2 treatment and changes in Bcl-2 and Bax expression is intriguing [53, 69]. Further, to explore the mechanism underlying the therapeutic effects of H_2 inhalation in TMT-induced mice, we assessed the expression of Bcl-2, and Bax protein. Our results showed that Bcl-2 expression was decreased in TMT Only group, while Bax expression was increased in TMT Only group. In contrast, H_2 treatment reverses the alteration of Bcl-2 and Bax. Together, these data indicate that H_2 inhalation ameliorates the TMT-induced toxicity and cognitive impairment in mice through inhibiting hippocampal apoptosis through Bcl-2 and Bax signaling pathways. Based on these overall findings, we can conclude that H_2 administration in TMT-induced mice improves cognitive function and neurotoxic damage due to its antioxidative and anti-inflammatory effects.

V. CONCLUSION

In summary, we evaluated the therapeutic effects of H_2 gas inhalation on neurotoxicity and cognitive impairment in TMT-injected C57BL/6 mice. Our results showed that H_2 gas inhalation effectively attenuated TMT-induced cognitive damage in mice. In addition, we found that H_2 effectively decreased TMT-induced OS and inflammatory markers, such as ROS, NO, MDA, and inflammatory cytokines such as G-CSF, IL- 1β, and TNF-α in the blood andbrain lysates of TMT-induced mice. In addition, H_2 increases VEGF levels in both the serum and brain in TMT-induced mice. Furthermore, H_2 inhalation decreased AD signaling protein markers such as Apo-E, Aβ-40, and p-tau in the TMT-induced brain. In addition, H_2 ameliorates the TMT-induced toxicity in mice through regulating hippocampal apoptosis through Bcl-2 and Bax signaling pathways. However, further molecular mechanistic studies on different signaling pathways are required to investigate the detailed therapeutic role of H_2 inhalation in TMT-induced neurotoxicity and cognitive damage. Taken together, our results strongly suggest that H_2 treatment might potentially be a future therapeutic candidate for neurodegenerative diseases by improving the cognitive abilities and controlling OS and inflammation-mediated neurodegeneration.

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VII. ABSTRACT IN KOREAN

Trimethyltin으로 유도된 신경독성 인지장애 마우스모델에서 수소 가스 흡입 처치의 치료효과

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산화 스트레스는 알츠하이머 병이나 인지기능 장애와 같은 다양한 신경퇴행성 질환들의 주요 발병요인이다. 본 연구는 trimethyltin(TMT)으로 신경 독성과 인지 장애를 일으킨 C57BL/6 마우스에게 수소가스 흡입시 치료 효과와 그 기전을 조사하였다. 생체효과 측정을 위해 생쥐를 4개군으로 나누었다: TMT를 처리하지 않은 정상군(NC), TMT만

처리한 대조군(TMT Only), TMT injection + lithium chloride 양성 대조군(PC), TMT injection + 2% H_2 흡입실험군(H_2). TMT는체중 kg 당 2.6mg을 1회 복강 주사하였고, 양성 대조를 위해 사용된 염화 리튬(lithium chloride)은 체중 kg 당 50mg을 1일 1회씩 2일 동안 복강 주사하였으며, 2% 수소가스는 1일 1회 30분씩 총 4주간 흡입시켰다. 인지능력을 측정하기 위하여 Y-maze를 이용한 행동반응 검사를 시행하였다. 산화스트레스 또는 알츠하이머와 관련된 검사지표로서 혈액과 뇌조직 샘플을 이용하여 활성산소(ROS), 산화질소(NO), 칼슘 이온(Ca2+), 말론디알데히드(MDA), 글루타티온 과산화효소(GPx), 카탈라아제, 염증성 시토카인, Apo-E, 아밀로이드 베타(amyloid-beta, Aβ)-40, p-tau, Bcl-2, Bax를 측정하였다. 연구 결과, TMT 처리 후 발작(seizure)이나 공간인지능력(spatial recognition memory)이 현저히 감소하였다. 그러나 수소를 흡입한 실험군에서는 대조군과 비교하여 기억력 감퇴가 줄었다. 또한 수소흡

입군은 Apo-E, Aβ-40, p-tau, Bax와 같은 알츠하이머 관련 지표들을 혈중과 뇌조직 내에서 감소시켰으며, ROS, NO, Ca^{2+}, MDA와 같은 산화스트레스 지표들을 억제하는 결과를 보여주었다. 마찬가지로 대표적인 항산화효소인 카탈라아제와 GPx의 활성이TMT 처리 후 현저히 증가하였으나 수소 가스 흡입 처리 후 TMT Only 대조군과 비교하여 혈액과 뇌조직 모두에서 유의하게 감소한 것으로 나타났다. 또한 과립구집락자극인자(granulocyte-colony stimulating factor, G-CSF), IL-6, tumor necrosis factor(TNF)-α와 같은 염증성 시토카인은 수소가스 흡입 후 유의하게 감소하였으며, Bcl-2와 vascular endothelial growth factor(VEGF) 단백질 발현은 수소 흡입 후 증가하였다. 결론적으로 TMT를 주사하여 화학적으로 신경독성을 일으킨 생쥐에게 2% 수소가스를 흡입하게 하였을 때 기억력이 향상되었고, 알츠하이머, 산화스트레스, 염증 등과 관련된 지표들이 혈중 및 뇌조직 내에서 감소되었다. 이런 결과는 수소 가스 흡

입이 인지기능장애를 보이는 신경퇴행성질환을 개선시키는데 효과가 있음을 강력히 시사한다. 그러나 TMT로 유발된 신경독성이나 인지장애에 대한 수소 흡입 효과를 규명하기 위한 기전 연구가 더 이루어져야 할 것으로 생각된다.

핵심단어: 수소 분자, 트리메틸틴, 산화스트레스, 알츠하이머 질환, 인지장애

VIII. PUBLICATION LIST

Jeong, E.-S.,Bajgai, J., You, I.-S., Rahman, M.-H., Fadriquela, A., Sharma, S., Kwon, H.-U., Lee, S.-L, Kim, C.-S., Lee, K.-J **(2021)**. Therapeutic Effects of Hydrogen Gas Inhalation on Trimethyltin-Induced Neurotoxicity and Cognitive Impairment in C57BL/6 Model. Int. J. Mol. Sci.2021.

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