INTRODUCTION

Vascular dementia (VaD), the second most common type of dementia after Alzheimer’s dementia, triggers cognitive impairment (dementia) attributable to cerebrovascular accident (CVD) [1]. When blood supply to the
brain is interrupted after CVD, cellular components of the central nervous system (CNS) are deprived of vital oxygen and glucose, causing damage to responsible CNS subfields, such as the hippocampus, associated with learning and memory [2].

There is evidence that ischemic insults finally cause VaD-associated neuronal death through intracellular accumulation of reactive oxygen species (ROS) [3]. In fact, under ischemic insults, deprived supplementation of oxygen and glucose elicits an increase in glutamate release from the axon terminals of presynaptic neurons, which in turn causes excessive calcium entry into dendritic spines of postsynaptic neurons [4]. Calcium then triggers ROS overproduction by affecting mitochondrial dysfunction [5]. Since excitotoxicity is inevitably linked to ROS accumulation, antioxidant treatment is likely to be an appropriate strategy for protecting against VaD [6].

Molecular hydrogen (H2) is an effective and bio-safe molecule with a wide mode of action for treating various ROS-related diseases, including VaD [7]. Although the vast majority of potential mechanisms of action remain to be elucidated, it has been revealed that H2 exerts its beneficial effects through antioxidant, anti-inflammatory, and anti-apoptotic effects, thus providing cytoprotection [8]. The protective effects of H2 on pathological conditions have been investigated using animal models of fibrotic heart disease [9], hepatic fibrosis [10], cerebral ischemia [11], radiation injury [12], and diabetes [13], in which intracellular accumulation of ROS is essentially involved in their pathogenesis.

There are several methods to administer H2, including inhaling gaseous hydrogen, drinking H2-rich water, and injecting H2-rich saline. Drinking H2-rich water attenuated stress-induced memory impairments in mice by reducing oxidative stress [14]. In addition, injection of H2-rich saline enhanced memory function in rats with amyloid-β-triggered dementia by reducing oxidative stress [15]. Moreover, inhalation of gaseous H2 during resuscitation rescued ischemic neuronal damage in a rat model of cardiac arrest [16]. An alternative to supplying H2 that has gained scientific attention, involves the reaction of metal hydrides with water. Although research trials on organisms are scarce, with this method, hydrogen can be directly generated on-site by the hydrolysis reaction of metal hydrides, e.g., magnesium hydride (MgH2), in a moist environment such as the alimentary tract [17]. The most important advantages of this reaction are that (i) the weight yield of the released hydrogen is high when water is included; in detail, 1 g of MgH2 produces 1820 cc of H2, (ii) the byproduct Mg(OH)2 is biocompatible, and (iii) the low cost of MgH2 [18-20]. Although pure MgH2 hydrolysis occurs extremely slowly, the kinetics and H2 yield can be strongly accelerated in the presence of acidic pH. As the pH of gastric acid is 1.5~3.5, we hypothesized that H2 can be effectively generated from MgH2 in situ, i.e., in the mammalian stomach lumen. In this study, we examined whether intragastric administration of MgH2 could suppress memory impairment and hippocampal neuronal death in a rat model of VaD.

**MATERIALS AND METHODS**

**Preparation of MgH2**

MgH2 was synthesized and kindly gifted by Solco Biomedical (Pyeong-Taek, Korea). Briefly, the two chemicals, magnesium (24.3 in atomic weight, 99.99% purity, Metal player, Incheon, Korea) and hydrogen (2 in atomic weight, 99.99% purity, Deokyang, Ulsan, Korea) were reacted in custom-made equipment (Reactor System, Solco biomedical) under high temperature (351~370°C) and high pressure (0.96 Mpa) for 90 h to yield 7.6 wt% (2/24.3 + 2 × 100) of H2 as described previously (#US8758643B2).

**Measurement of the dissolved H2 concentration**

Five-hundred milligrams of MgH2 powder was dissolved in 200 mL normal saline, and the pH adjusted to 2.5, to mimic intragastric conditions, while continuous stirring at 37°C was done. The H2 concentration of the mixture was measured using a hydrogen concentration measuring instrument (KM2100 DH, Kyoei Denshi Kenkyuujo, Japan) at pre-determined times.

**Animals**

A total of 32 male Sprague Dawley rats (8 weeks, 200~250 g) were purchased from Samtako (Osan, Korea). The rats were housed in an environmentally controlled room at constant temperature (21~23°C) and relative humidity (40~60%) under a 12-h light/dark cycle. All rats had free access to water and food. Experiments were conducted in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ (National Institutes of Health publi-
cation, 8th Edition, 2011) [21]. The animal experiments in this study were approved by the Institutional Animal Care and Use Committee (approved protocol number: P-18-14-A-01) of Konyang University (Daejeon, Korea).

**Experimental design**

All rats were randomly assigned to 4 groups (n = 8 per group) and then treated differently as follows: SO, a group treated with normal saline (0.9% w/v of sodium chloride) used as a vehicle and underwent sham-operation; VEH, a group treated with a vehicle and underwent 2 vessel occlusion and hypovolemia operation (2VO/H); MH-L, a group treated with a low dose (5 mg/kg) of MgH₂ and 2VO/H; and MH-H, a group treated with a high dose (15 mg/kg) of MgH₂ and 2VO/H. Using these rats, three different behavioral tests, i.e., the Y-maze, Barnes maze, and passive avoidance tests, were conducted at the indicated times. For histological analyses, all rats were sacrificed immediately after finishing the three behavioral tests.

**Two vessel occlusion and hypovolemia (2VO/H) operation**

To establish an animal model of VaD, 2VO/H was employed using the method previously described by Smith et al. [22] with some modifications. For this, anesthesia was achieved with 5% isoflurane in 70% N₂O and 30% O₂ and maintained during the operation at a level of 1.5~2% isoflurane under spontaneous respiration. Throughout the operation, the rectal temperature was controlled at 37°C with a heating pad. The left femoral artery was exposed and catheterized with a PE-50 catheter to allow future withdrawal of blood to cause hypovolemia. The right jugular vein was isolated and injected with 500 U/kg heparin dissolved in 100 U/mL with 0.9% saline. After exposing the bilateral common carotid arteries (CCAs), these two vessels were permanently ligated with a 4-0 nylon suture. Next, blood was gradually withdrawn from the femoral artery via a catheter to achieve a reduction of cerebral blood flow (CBF). When the relative CBF (rCBF) value, measured by laser Doppler flowmetry (Periflux5000; PerimedAB, Järfalla-Stockholm, Sweden), was approximately <20% of baseline, withdrawal of blood was stopped for 10 min to maintain an ischemic period. Throughout this period, the syringe filled with pulled blood was kept in a shaking water bath at 37°C to prevent coagulation. Subsequently, the blood was reinfused, rapidly returning the CBF to baseline. After reaching a plateau, the animals regained consciousness and were maintained in their home cage until further experimentation.

**Y-maze test**

The Y-maze test was employed as the first tool to assess memory performance. For this, the rats were initially introduced to the center of the matte black plastic maze with three arms at 120-degree intervals, which were 50 cm long, 15 cm wide, and 30 cm high. The sequence and number of arm entries were monitored for 8 min period. To calculate the percentage of spontaneous alternation, the following formula was used: spontaneous alternation (%) = ([number of alternations]/(total number of arm entries-2)] × 100. The number of total arm entries also served as an indicator of locomotor activity. After each rat was tested, the maze was cleaned using 70% ethanol.

**Barnes maze test**

The Barnes maze test was employed as the second tool
to assess memory performance. For this, a 100-cm-high circular platform with a diameter of 122 cm was used. There are 20 holes located around the perimeter with a black escape box (20×15×12 cm) placed under one of these. The task was divided into two stages: stage 1, training trials on postoperative days (POD) 2, 3, 4, and 5; and stage 2, a probe test on POD 6. During the training trials, the animal was placed on the table and given 120 s to find and enter the escape box, with bright lights as aversive stimuli. Each animal performed one trial per day during this stage. On the probe test day, the escape box was removed, and the time spent in the quadrant where the escape box was originally located was recorded. The explorative behavior of individual rats during stages 1 and 2 was recorded and analyzed with the aid of a video camera connected to an EthoVision XT9 system (Noldus, Wageningen, Netherlands). After each rat was tested, the circular platform and escape box were cleaned using 70% ethanol.

**Passive avoidance test**

The passive avoidance test was employed as the third tool to assess memory performance. The test apparatus was equipped with two chambers, i.e., an illuminated chamber and a dark chamber, each measuring 25×20×25 cm. A lamp (50 W) was used as the source of light in the illuminated chamber. Each test involved two separate sessions: a trial session and a test session. During the trial session (POD 7), the rats were initially placed in the illuminated chamber. The “trial latency” time, once the rat had entered the dark compartment, was measured using a stopwatch. This measurement was triplicated and at the last entry of the rat to the dark chamber, an electrical shock (0.5 mA) for 3 s was delivered through stainless steel rods. A test session was performed 24 h following the trial session, and the “escape latency” times to re-enter the dark chamber were measured up to 180 s. After each rat was tested, the walls of the chambers were cleaned using 70% ethanol.

**Cresyl violet staining**

On POD 8, rats were transcardially perfused with 4% paraformaldehyde (PFA). Their brains were isolated and post-fixed with 4% PFA, dehydrated with a graded ethanol series, embedded in paraffin, and serially sectioned at 5-μm thickness using a microtome (RM2255, Leica, Nussloch, Germany). The two slides randomly selected from the hippocampus-bearing tissue collections per rat were de-paraffinized in xylene, hydrated in a decreasing ethanol gradient series, and washed twice in distilled water. The slides were then stained with a 0.1% Cresyl violet solution (Sigma-Aldrich, St. Louis, MO, USA). Each hippocampal Cornu Ammonis (CA) 1 region was photographed at ×400 magnification using a digital camera connected to a light microscope (DM4, Leica). The number of intact neurons localized in the CA1 subfield at 300 μm in width was counted and averaged in the photographs. At this time, only neurons with clear nuclei and large cell bodies were considered morphologically intact.

**Statistical analyses**

All data are presented as the mean± standard error of the mean (SEM). Data comparison between groups was performed using one-way ANOVA in GraphPad (GraphPad Prism 5; GraphPad Software, Inc., San Diego, CA, USA). \( P<0.05 \) indicated a statistically significant difference.

**RESULTS**

**MgH₂ continuously generated \( \text{H}_2 \) in an acidic aqueous environment**

\( \text{MgH}_2 \) was first tested for its time-dependent \( \text{H}_2 \)-generating effects under conditions mimicking the stomach lumen. As shown in Fig. 2, 500 mg \( \text{MgH}_2 \) powder could generate 2000 ppb \( \text{H}_2 \) immediately after dissolution in normal saline (pH 2.5) at physiological temperature (37°C). Approximately half of the initial amount of \( \text{H}_2 \) was released from \( \text{MgH}_2 \) by 7.5 days, and the complete depletion of releasable \( \text{H}_2 \) was observed by 21 days. These observations indicate that \( \text{MgH}_2 \) can continuously generate \( \text{H}_2 \) in an acidic environment such as the intragastric lumen. After confirming sustained generation of \( \text{H}_2 \) from \( \text{MgH}_2 \), rats were treated with \( \text{MgH}_2 \) or normal saline as a vehicle using a daily regimen for 14 days before 2VO/H operation.

**MgH₂ attenuated memory impairment in VaD rats**

Thereafter, we investigated whether intragastric admin-
istration of MgH₂ can prevent memory impairment, an essential feature in VaD patients. For this, we assessed the effects of chronic intake of MgH₂ on VaD-associated memory impairment using rats who underwent 2VO/H, a surgical model of VaD. Three different behavioral analyses, that is, Y-maze, Barnes maze, and passive avoidance tests, were employed to assess memory employment in the rat groups. The results from the Y-maze test indicated that the VEH group showed remarkable impairments in memory function, as shown by spontaneous alternation ($p^{***} < 0.001$ vs. CTRL; Fig. 3A). However, both the MH-L and MH-H groups showed a significant increase in spontaneous alternation behavior when compared with the VEH group ($p^{<} < 0.05$ and $p^{##} < 0.01$ vs. VEH, respectively), although not dose-dependent. Neither 2VO/H nor MgH₂ treatment significantly affected locomotion, as demonstrated by total arm entry (Fig. 3B). The results from the Barnes maze test demonstrated that all the groups spent similar amounts of time exploring the escape box on the first day of the training trials (Fig. 4A-F). However, on the 2nd, 3rd, and 4th day of training trials, the VEH group spent a longer time and moved a longer total distance to find the escape box when compared with the SO group ($p^{***} < 0.001$ vs. SO). Interestingly, the escape latency and the moving distance were significantly decreased in the MH-L and MH-H groups when compared with the VEH group at all times in the training trials except on the 1st day ($p^{<} < 0.05$ and $p^{<} < 0.05$ vs. VEH, respectively). Neither 2VO/H nor MgH₂ treatments significantly affected locomotion, as shown in the velocity plots (Fig. 4G). During the probe test, the VEH group spent less time in the target quadrant where the escape box was formerly located ($p^{**} < 0.01$ vs. SO), suggesting that 2VO/H triggered memory deficits in rats (Fig. 5A and 5B). Interestingly, this deficit was prevented in the MH-L and MH-H groups, although dose-independently. Finally, the effect of MgH₂ on AD-associated memory impairment was assessed using the passive avoidance test. The results showed that the VEH group developed a significant impairment in memory retention when compared with the SO group, as evidenced by the shorter escape latencies ($p^{**} < 0.01$ vs. SO; Fig. 6A) in the test session. However, the escape latencies of the MH-L and MH-H groups were significantly longer than those of the VEH group ($p^{<} < 0.05$ and $p^{##} < 0.01$, respectively), and these changes were dose-dependent ($p < 0.05$). As shown in Fig. 6B, neither 2VO/H nor MgH₂ affected the trial latencies assessed.

Fig. 2. MgH₂ dissolution curve. The vehicle used was acidic normal saline (0.9% w/v sodium chloride). 500 mg MgH₂ powder was dissolved in 200 mL normal saline and the pH adjusted to 2.5, thus mimicking the gastric lumen. H₂ concentration was measured using a hydrogen meter at pre-determined times.

Fig. 3. Effects of MgH₂ on spontaneous alternation in rats with vascular dementia. These were tested using the Y-maze test. The average spontaneous alternation rate (A) and the number of total arm entries for > 8 min (B) were measured in rats of different groups. Values are presented as the mean ± SEM ($p^{***} < 0.001$ vs. SO; $p^{<} < 0.05$ and $p^{##} < 0.01$ vs. VEH; N.S, no significance).
Fig. 4. The effects of MgH\(_2\) on escape latency in rats with vascular dementia. These were tested using the Barnes maze training trial. Representative tracking plots of SO (A), VEH (B), MH-L (C), and MH-H groups (D) on the 1\(^{st}\) and 4\(^{th}\) day of the Barnes maze training trial. Moving distance (E), escape latency (F), and escape velocity (G) for up to 120 s were recorded. Values are presented as the mean ± SEM (\(p^{***} < 0.001\) vs. SO; \(p^\# < 0.05\), MH-L vs. VEH; \(p^\$ < 0.05\), MH-H vs. VEH).

Fig. 5. The effects of MgH\(_2\) on time spent in the target quadrant. These were tested using the Barnes maze probe test. Representative tracking plots of SO, VEH, MH-L, and MH-H groups (A) are shown. The time spent in the target quadrant during the probe test was recorded and statistically analyzed. Values are presented as the mean ± SEM (\(p^{**} < 0.01\) vs. SO; \(p^\# < 0.05\) vs. VEH; N.S, no significance).
during the training session, indicating that seeking behavior or sensitivity to light was not affected by 2VO/H and MgH₂. Together, these results suggest that MgH₂ significantly ameliorated spatial learning and memory deficits in VaD rats.

**MgH₂ attenuates deteriorations in hippocampal structures in VaD rats**

Next, we investigated whether MgH₂ can prevent morphological deterioration in the hippocampal structure, an essential feature in either 2VO/H-induced VD rats or VD
patients. For this, using Cresyl-violet stain, we assessed the effects of 14 days of pretreatment with MgH2 on 2VO/H-induced loss of pyramidal neurons in the hippocampal CA1 region. On POD 8, the VEH group showed a dramatic decrease in the number of hippocampal CA1 neurons when compared with that of the SO group, as shown in Fig. 7A and 7B (*p* < 0.001 vs. SO). In the VEH group, in addition to the loss of neuron number, the neurons undergoing apoptosis, characterized by pyknotic nuclei and shrunken cytoplasm, were abundant in the stratum pyramidale (SP) of the hippocampal CA1 region (black arrows in Fig. 7A). However, the number of surviving neurons was significantly increased in the MH-L and MH-H groups (p<0.001 vs. VEH), and this change was dose-dependent (p<0.001). Collectively, these results suggest that intragastric administration of MgH2 could prevent VaD-associated hippocampal neuronal loss.

**DISCUSSION**

Molecular hydrogen (H2), which is nonpolar, small, and electrically neutral, can rapidly penetrate all cell membranes; as such, it can easily cross the blood-brain barrier. In 2007, Ohsawa et al. reported that gaseous H2 acts as a therapeutic antioxidant in cells by selectively scavenging ROS and reactive nitrous species (RNS), such as the hydroxyl radical (‘OH) and peroxynitrite (ONOO-), thus conferring cytoprotection against oxidative damage [23]. Since this strong therapeutic effect of H2 on a rodent model of cerebral ischemia was reported, multiple studies showed that several experimental models of pathology can benefit from H2. An increasing number of studies revealed the therapeutic effects of H2 in various CNS disease models, and that it is due to its anti-oxidative, anti-apoptotic, and anti-inflammatory activities [24]. However, the potential molecular mechanisms are still unclear, and some results remain controversial, thus requiring further research. Moreover, since the current findings are mainly based on animal experiments, whether these findings can translate to humans remain unanswered. Therefore, further human studies are needed to validate the findings seen in rodents. Nevertheless, the advantages of H2 have provided important means and optimistic prospects for preventing CNS diseases.

The right amount of hydrogen supplementation might have certain benefits for treating various pathologies, including VaD. H2-rich water has received widespread attention in the international market in recent years. However, the widespread use of H2-rich water is restricted by the low solubility of H2 [25]. The hydrogen content in H2-rich water is generally approximately 0.1–1 ppm, with the highest concentration known to be approximately 5 ppm [26]. To overcome the issue of low H2 concentration, we used MgH2 as the H2 donor in this study. When compared with previous reports that used H2-rich water as an H2 donor, the present trial has the following beneficial effects: 1) a supplement of magnesium and hydrogen preparation can simultaneously supplement magnesium and H2, which can neutralize ROS and RNS; 2) unlike H2-rich water, limited by the low solubility of hydrogen, the amount of H2 supplemented by our preparation is greatly improved in the acidic environment of the stomach; 3) the reaction metabolites (Mg2+, 2OH, and 2H2) have no toxic side effects or adverse reactions and, therefore, are safe and reliable; and 4) since MgH2 is a powder, its production cost is low; therefore, it is suitable for large-scale production and storage.

Using this formulation, H2 could be supplied in a sustainable manner; therefore, we tested its effects on an in vivo experimental model of VaD achieved surgically, i.e., 2VO/H. Traditionally, artificial interruption of all four vessels that supply the brain, both CCAs and vertebral arteries, the so-called 4 vessel occlusion technique (4-VO), has been widely used to produce a rat model of VaD [27]. While both CCA occlusions can be accomplished using a minimally invasive ventral neck incision and application of aneurysm clips for the desired period, the occlusion of both vertebral arteries can be technically difficult, as they are located within the transverse foramina of the 2nd cervical vertebra, which is hardly visible under gross inspection [28]. In contrast with 4-VO, both CCAs occlusion coupled with systemic reduction of mean arterial blood pressure leading to a significant decrease in CBF, i.e., the so-called 2VO/H model, can produce ischemia throughout the forebrain, resulting in a pattern of brain damage closely mimicking CVA seen in VaD. In our preliminary experiments and from the results obtained in this study, POD 8 represents the optimal time at which CA1 hippocampal neuronal death can be quantified using Cresyl violet staining. Thus, all remaining cells at POD 8 can be assumed to be viable cells, thus providing an index of brain damage or rescue after certain interventions.

This study provides evidence that MgH2 supplementa-
tion can exert anti-VaD effects, attenuating memory deficit and hippocampal neuronal death, the two essential features of VaD. However, the major limitations of this study are still remained. First, we could not rule out the possibility of involvement of magnesium effect on MgH2-triggered anti-VaD effect. In fact, a few studies clearly demonstrated that magnesium supplement attenuated the VaD-associated symptoms [29] and risks [30] in human. Thus, further studies for dissecting out of the possible involvement of magnesium from MgH2-induced protection against VaD is remained to be fulfilled. Second, this study lacks detailed explanations regarding mechanisms underlying MgH2-mediated anti-VaD effects in vivo. Considering that VaD involves complex pathological cascades, including a series of molecular and cellular events such as apoptosis, neuroinflammation [31], and mitochondrial dysfunction other than ROS-associated oxidative damage [32], more advanced and well-designed studies should be performed in the future to provide a more detailed explanation about the specific mechanisms underlying MgH2-mediated protection against VaD.

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