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Original Article

Targeting hepcidin improves cognitive impairment and reduces iron deposition in a diabetic rat model

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Abstract: Cognitive impairment is a common complication of type 2 diabetes mellitus (T2DM) that may be related to iron deposition in the brain. Hepcidin is expressed in the brain and has the ability to regulate iron. Therefore, this study explored the role of hepcidin in hippocampal iron deposition and cognitive impairment in T2DM. The effects of a recombinant adeno-associated virus targeting hepcidin (AAV-hepcidin) for hippocampal iron content and cognitive function were investigated in a T2DM rat model induced by streptozotocin and a high-fat diet. Adult male rats (n = 32) were categorized as either C-saline (normal control), M-saline (T2DM), M-blank (AAV-blank + T2DM), or M-hepcidin (AAV-hepcidin + T2DM). Hippocampal iron content was assessed using quantitative susceptibility mapping. Morris water maze (MWM) testing was used to assess the cognitive function. Magnetic resonance imaging indicated that hippocampal susceptibility values were significantly increased bilaterally in T2DM rats compared with controls ($P = 0.044$, $P = 0.043$). Compared with the M-blank group, the M-hepcidin group exhibited significantly decreased hippocampal susceptibility values bilaterally ($P = 0.007$, $P = 0.030$). Compared with the M-saline group, susceptibility values from left hippocampus in the M-hepcidin group were significantly reduced ($P = 0.002$). MWM results showed that the performance of T2DM rats was significantly decreased from that of control rats. Compared with the M-saline and M-blank groups, the performance of the M-hepcidin group was significantly increased. These studies demonstrate that T2DM rats developed cognitive impairment and iron deposits in the hippocampus, both of which were improved by AAV-hepcidin administration.

Keywords: Type 2 diabetes mellitus, hepcidin, iron deposition, cognitive impairment, magnetic resonance imaging, quantitative susceptibility mapping

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by hyperglycemia and insulin resistance, and it is becoming increasingly common. Importantly, studies have shown that T2DM is associated with cognitive impairment [1]. For example, patients with T2DM may have memory, attention, and executive function deficits [2]. Learning and memory impairments have also been found in studies of T2DM animal models [3]. Furthermore, T2DM increases the risk of Alzheimer's disease and dementia [4, 5]. Because the specific reasons for cognitive impairment remain obscure, elucidating the underlying mechanisms of T2DM-induced impairment would be very significant for its prevention and treatment.

The etiology of cognitive dysfunction in T2DM may be multifactorial, and emerging evidence indicates that it is related to iron overload in the body and brain, especially in the hippocampus [6, 7]. Iron is an important element for maintaining normal neurological function [8], but iron accumulation in cells may induce cell damage by catalyzing the Fenton reaction to produce reactive oxygen species. In addition, the

central nervous system is extremely susceptible to oxidative stress [9]. Thus, iron deposition in the central nervous system may cause oxidation and modification of DNA, proteins, and lipids, leading to neural damage [10]. The subsequent loss of neurons can then lead to a gradual loss of functional capacity [9]. There is evidence that oxidative stress caused by brain iron deposition is one of the basic causes of neuronal death in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [11]. However, the underlying mechanism responsible for iron deposition in T2DM is still unclear.

Dysregulation of hepcidin expression may be responsible for diabetic iron deposition [12]. Hepcidin is an antimicrobial peptide that plays an essential role in iron homeostasis and is widely distributed in the brain and liver [13]. Hepcidin inhibits the release of iron from macrophages and hepatocytes by inducing degradation of its receptor, the cellular iron exporter ferroportin, thereby regulating intestinal iron absorption and plasma iron concentration as well as reducing the tissue iron content [14]. Moreover, hepcidin deficiency is associated with iron deposition in the body. For instance, hemochromatosis is a hereditary condition characterized by systemic iron overload caused by a lack of hepcidin [15]. Recent studies have found that increasing hepcidin can decrease the brain's iron content in iron-overloaded rats by downregulating iron transporters, thereby reducing iron-mediated oxidative stress [16, 17]. Although hepcidin therapy has been used effectively for the treatment of iron overload, it is unclear whether an increased level of hepcidin could improve cerebral iron deposition and cognitive impairment in T2DM.

We hypothesized that a deficiency of hepcidin in T2DM may lead to iron deposition in the brain, which in turn leads to cognitive impairment. This study was designed to investigate the role of hepcidin in cognitive impairment, and brain iron deposition in T2DM, using magnetic resonance imaging (MRI) and the Morris water maze (MWM) test. Iron content in the brain was assessed using quantitative susceptibility mapping (QSM), and cognitive function was evaluated using the MWM test in a T2DM rat model induced by streptozotocin (STZ) and a high-fat diet (HFD). The upregulation of brain hepcidin was achieved using a recombinant adeno-associated virus targeting hepcidin (AAV-hepcidin). We hypothesized that the development of T2DM would cause cognitive impairment and iron overload in the hippocampus, and that AAV-hepcidin would suppress brain iron disposition and improve cognitive impairment in T2DM rats.

Materials and methods

Materials and animals

Male Sprague-Dawley rats (120-150 g; 5 weeks old) were obtained from the Laboratory Animal Center of the Third Military Medical University, maintained in a constant temperature and humidity environment using a 12-h light/dark cycle, with access to food and water *ad libitum*. All experimental procedures were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University in Chongqing, China.

Animal model and groups

Thirty-two rats were randomly divided into four groups: a normal control group (buffer + saline, termed *C-saline*), a T2DM-only group (STZ + saline, termed *M-saline*), an AAV-blank + T2DM group (STZ + AAV-blank, termed *M-blank*) and an AAV-hepcidin + T2DM group (STZ + AAV-hepcidin, termed *M-hepcidin*). C-saline rats were fed a standard diet for 8 weeks, and M-saline, M-blank and M-hepcidin rats were fed an HFD diet for 8 weeks. T2DM was induced by intraperitoneal injection of STZ (30 mg/kg; Sigma-Aldrich, St. Louis, MO) under fasting conditions. C-saline rats received same volume vehicle-only injections (citrate buffer). On the fifth day after STZ injections, rats with fasting blood glucose levels >11.1 mmol/L were identified as diabetic. After confirmation of the model, rats were fed for another 16 weeks, and serum parameters such as fasting blood glucose and insulin were measured at the end of all experiments.

Intracerebroventricular injections and AAV construction

Virus injections were performed 12 weeks after T2DM model was established. Rats were treated with 3 μL of either AAV-hepcidin (M-hepcidin group), AAV-blank (M-blank group), or saline

(C-saline group and M-saline group) administered to the right hemisphere through an intracerebroventricular injection. Injections were performed using a stereotaxic frame to guide a micropipette into the right lateral cerebral ventricle (bregma, -0.8 mm; lateral, 1.5 mm; ventral, -3.5 mm) according to a standard stereotaxic atlas. The micropipette was withdrawn 10 min after injection, and the incision was closed with sutures.

A recombinant adeno-associated virus serotype 2/9 vector (rAAV2/9) expressing cytomegalovirus (CMV)-driven enhanced green fluorescent protein (EGFP) (rAAV-CMV-EGFP-WPRE-pA) was used. To overexpress hepcidin, rAAV-CMV-Hepc-EGFP-WPRE-pA (termed *AAV-hepcidin*) targeting the rat Hepc gene (GenBank: NM_053469) was used. As a negative control, rAAV-(empty vector)-CMV-EGFP-WPRE-pA (termed *AAV-blank*) was used. The AAV virus vector was designed and constructed by BrainVTA (Wuhan, China) using standard methods.

Morris water maze

To assess cognitive impairment, spatial learning and memory were tested using a Morris water maze (MWM; Third Military Medical University, Chongqing, China) after 5 weeks of intervention. Rats in the M-hepcidin, M-blank, M-saline, and C-saline groups (n = 8 per group) were subjected to the MWM test. The maze consists of a stainless-steel circular tank 180 cm in diameter, 50 cm in height, divided into four quadrants, filled with water (22°C) to a depth of 40 cm, and placed in a room with external visual cues. A 10-cm diameter platform, submerged 1 cm below the water surface, was placed in the third quadrant. Testing was carried out according to the protocol of Vorhees and Williams [18]. Briefly, for the positioning navigation trials, animals were trained for 5 days (four trials each day). For each trial, the rat was placed randomly in one of four quadrants facing the tank wall and allowed to swim for a period of 60 s to find the hidden platform. Rats failing to find the platform within 60 s were guided and placed on the platform for 10 s. The time required (latency) to find the hidden platform and the time spent in the third quadrant were both recorded. The probe trial was administered 24 h after the final positioning navigation trial. For each probe trial, the rat was placed in the first quadrant and allowed to swim for 60 s with the platform removed. The number of crossings through the platform zone and the time spent in the third quadrant were both recorded.

Magnetic resonance imaging

MRI measurements were performed in M-hepcidin, M-blank, M-saline, and C-saline rats (n = 8 per group) after the MWM. MRI data were obtained using a 7T scanner (Bruker, Germany) with a birdcage-type coil as the transmitter and a quadrature half-volume coil as the receiver. During MRI measurements, rats were fixed with stereotactic ear bars and anesthetized with medical air (1.0 L/min) and isoflurane (2-2.5%). Susceptibility-weighted images (SWI) were obtained using a high-resolution 3D gradient-echo sequence. The acquisition parameters included TR/TE = 488/12 ms, flip angle = 40°, slice thickness = 1 mm, FOV = 384 × 384 cm, pixel bandwidth = 77 Hz/pixel, and matrix size = 384 × 384. T2 images were obtained and analyzed to rule out brain abnormalities, such as space-occupying lesions or subdural hematomas. This sequence was not systematically analyzed, and therefore was not reported in detail.

Image processing and analysis

Signal Processing in Nuclear Magnetic Resonance (SPIN; MR Innovations Inc., Detroit, MI, USA) and Susceptibility Mapping and Phase Artifacts Removal Toolbox (SMART; Detroit, MI, USA) software were used for data processing and measurements. Five steps were used for image processing based on the Haacke et al. protocol [19]. First, skull stripping was used to remove noise outside the brain. Second, phase unwrapping was performed by using a Laplacian operator. Third, a high-pass filter of 32 pixels was applied for background field removal. Fourth, spurious phase removal was performed. Fifth, an iterative thresholded k-space division algorithm was used. Regions of interest (ROI) were manually drawn on two consecutive slices in the center of the hippocampus (**Figure 1**).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Differences between the C-saline group and M-saline group were

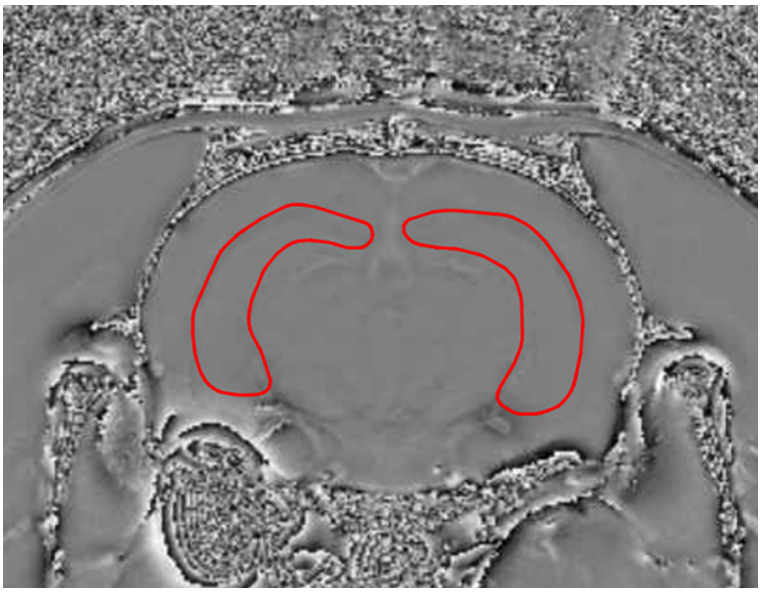

Figure 1. Regions of interest (ROI) were manually indicated on quantitative susceptibility mapping (QSM) images.

Table 1. Serum parameters in the C-saline and M-saline groups

Groups	M-saline (n = 6)	C-saline (n = 6)	*p* value
Glucose	21.20±0.94	4.85±0.15	0.000***
Insulin	10.62±0.74	8.10±0.28	0.017*
Insulin resistance index	10.08±0.99	1.75±0.09	0.000***
Ferritin	17.75±0.7	13.15±0.55	0.000***

*, $P < 0.05$, ***, $P < 0.001$.

determined by two-sample t tests. Differences among the M-hepcidin, M-blank, and M-saline groups were determined by a one-way ANOVA (analysis of variance) followed by post hoc least significant difference tests. A value of $P < 0.05$ was considered significant.

Results

Stability of the T2DM model

Measurements of glucose, insulin, and ferritin levels showed that the HFD and the STZ injection induced hyperglycemia, insulin resistance, and significantly increased serum ferritin concentrations in T2DM rats, indicating the stability of the T2DM model and an increase in body iron content in T2DM rats ($P = 0.000$; **Table 1**). Compared with M-blank and M-saline rats, M-hepcidin rats exhibited significantly decreased serum ferritin levels, indicating that AAV-hepcidin administration reduced iron content in the T2DM rats ($P = 0.000$; **Table 2**). There was no difference in ferritin levels between the M-blank group and the M-saline group.

Spatial learning and memory were impaired in T2DM rats

To investigate whether T2DM rats developed impaired spatial learning and memory, the latency to reach the platform during the positioning navigation trial, the number of crossings through the platform zone during the probe trial, and the time spent in the target quadrant during the probe trial were analyzed (**Table 3**). The gradual decrease in escape latency indicated that control rats quickly learned to reach the platform compared with T2DM rats (**Figure 2A**). The number of crossings through the platform zone in T2DM rats was significantly less than the number in control rats ($P = 0.009$; **Figure 2B**). Compared with the control group, the M-saline group exhibited significantly reduced time spent in the correct quadrant ($P = 0.016$; **Figure 2C**). These data demonstrated that learning and memory impairment occurred in T2DM rats.

AAV-hepcidin improved spatial learning and memory impairment in T2DM rats

To study whether AAV-hepcidin improved spatial learning and memory impairment, the escape latency during the positioning navigation trial, the number of crossings through the platform zone during the probe trial, and the time spent in the target quadrant during the probe trial in three groups of rats were analyzed (**Table 4**). The gradual decrease in escape latency in the M-hepcidin group indicated better learning ability compared with the M-blank and M-saline groups (**Figure 3A**). The ANOVA results indicated that the number of crossings through the platform zone was significantly different among the three groups. Rats in the

Table 2. Serum parameters for the three T2DM groups

Groups	M-hepcidin (n = 6)	M-blank (n = 6)	M-saline (n = 6)	*p* value	*p* value for post hoc analysis		
					a	b	c
Glucose	22.05±2.06	22.07±1.29	21.20±0.94	0.898	0.994	0.695	0.689
Insulin	9.14±0.22	9.74±0.3	10.62±0.74	0.121	0.389	0.044	0.21
Insulin resistance index	8.96±0.85	9.52±0.56	10.08±0.99	0.633	0.632	0.347	0.637
Ferritin	13.61±0.5	17.13±0.24	17.75±0.7	0.000***	0.000***	0.000***	0.404

[a]Differences between the M-hepcidin and M-blank groups. [b]Differences between the M-hepcidin and M-saline groups. [c]Differences between the M-blank and M-saline groups. ***, $P < 0.001$.

Table 3. Results of the Morris water maze test in the C-saline and M-saline groups

Groups	M-saline (n = 8)	C-saline (n = 8)	*p* value
Day 1 (s)	36.23±6.20	43.49±3.03	0.311
Day 2 (s)	32.62±4.52	32.78±6.6	0.985
Day 3 (s)	34.36±6.19	28.34±4.68	0.451
Day 4 (s)	32.67±6.96	15.89±2.44	0.039*
Day 5 (s)	32.62±4.89	16.99±2.66	0.014*
Crossings	1.63±0.46	3.13±0.3	0.009*
Time in the target quadrant (s)	10.36±2.05	18.68±1.85	0.016*

*, $P < 0.05$.

M-hepcidin group made significantly more crossings through the platform zone than both M-blank and M-saline group rats ($P = 0.015$, $P = 0.026$; **Figure 3B**). There was no statistical difference between M-blank and M-saline group rats. Compared with M-saline rats, rats in the M-hepcidin group spent more time in the correct quadrant, but this difference was not statistically significant (**Figure 3C**). These results demonstrated that learning and memory impairment in T2DM rats were improved after AAV-hepcidin administration.

Hippocampal iron deposition in T2DM rats

The hippocampal susceptibility values for the C-saline group and the M-saline group were -8.93 and 4.645 ppb ($\times 10^{-9}$), respectively. Bilateral susceptibility values for the hippocampus were significantly different between the C-saline and M-saline groups. Compared with the C-saline group, the susceptibility values of the M-saline group in the left and right hippocampus were significantly increased ($P = 0.044$, $P = 0.043$; **Figure 4A**, **4B**). These data indicated significantly higher iron concentrations in the hippocampus of T2DM rats compared with control rats, demonstrating that diabetic rats developed hippocampal iron deposition.

AAV-hepcidin improved hippocampal iron deposition in T2DM rats

The susceptibility values for the hippocampus of the M-hepcidin, M-blank, and M-saline groups were -0.685, 5.05, and 4.645 ppb ($\times 10^{-9}$), respectively. Analysis of variance showed that there were statistically significant differences in the bilateral hippocampal susceptibility values among the three groups. The hippocampal susceptibility values of the M-hepcidin group were significantly decreased compared with M-saline and M-blank group. M-hepcidin group rats exhibited significantly reduced susceptibility values in their left hippocampus compared with both M-saline and M-blank group rats ($P = 0.002$, $P = 0.007$; **Figure 5A**). Compared with M-blank group rats, the M-hepcidin group exhibited significantly decreased susceptibility values in the right hippocampus ($P = 0.030$; **Figure 5B**). There were no significant differences in bilateral hippocampal susceptibility values between the M-blank and M-saline groups. These data showed lower iron concentrations in the hippocampus of M-hepcidin group rats compared with M-blank and M-saline rats, suggesting that hepcidin therapy reduced iron deposition in T2DM.

Discussion

Cognitive dysfunction is a common clinical symptom of diabetes. T2DM is accompanied by a wide range of cognitive impairments, mainly in terms of learning and memory [20]. There is increasing recognition that iron accumulation in the brain is associated with cognitive dys-

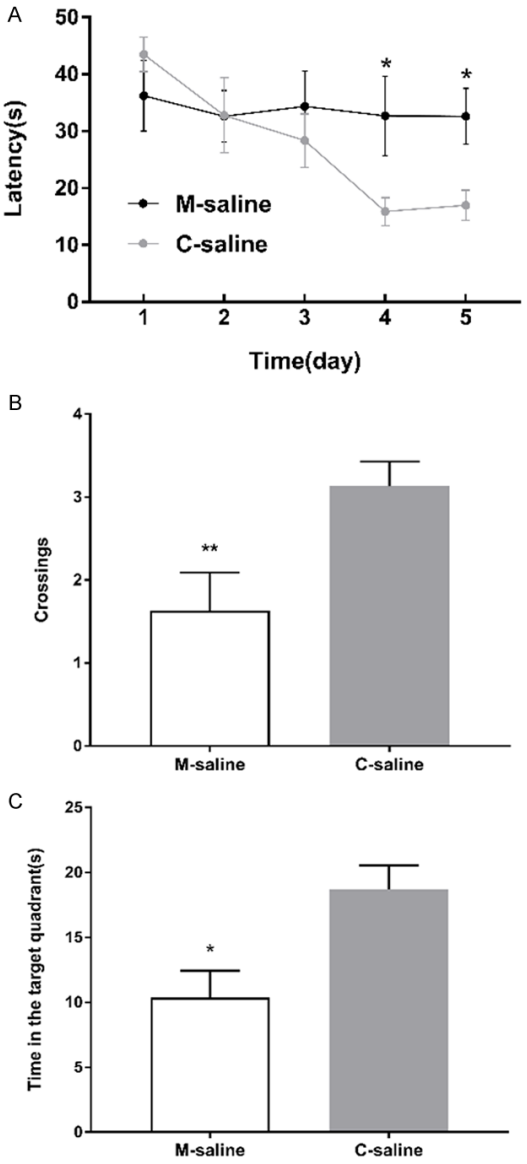

Figure 2. Spatial learning and memory were impaired in T2DM rats. A. Escape latency during the positioning navigation trial, * = significantly different from the C-saline group, $P < 0.05$. B. Number of crossings through the platform zone during the probe trial, ** = significantly different from the C-saline group, $P < 0.01$. C. Time spent in the target quadrant during the probe trial, * = significantly different from the C-saline group, $P < 0.05$.

function [21] and may lead to a 1.5-2.5-fold increase in the risk of dementia associated with obesity-related T2DM [1]. Although cognitive impairment may be caused by iron deposits in T2DM, a specific mechanism has not been identified [6, 22].

In the current study, the brain iron content of rats was analyzed by QSM. This method has been successfully used to study iron levels in the brain and has been shown to correlate well with histopathological data. Previous QSM studies have analyzed the brain iron content of the elderly, and the results were consistent with levels determined at autopsy [23]. In the present study, iron content data were obtained by manually mapping hippocampal ROIs. Compared with control rats, M-saline group rats showed increased bilateral susceptibility values in their hippocampus, demonstrating that hippocampal iron deposition occurred in T2DM. In addition, M-saline group rats exhibited significantly increased serum ferritin levels compared with rats in the C-saline group. M-hepcidin group rats exhibited significantly decreased serum ferritin levels compared with both M-blank and M-saline rats. Serum ferritin is the most commonly used index for body iron content in epidemiological research. The present results have shown both an increase in iron content in T2DM rats and its reduction by AAV-hepcidin administration. Although studies have shown that serum ferritin may also be elevated as a result of inflammation, liver disease and cancer, reflecting inaccurate iron levels [24]. Recently, increases in both hepatic and serum iron content as well as a significant increase in intestinal iron absorption have also been reported using a rat model of T2DM [25]. Similarly, clinical investigations have reported that patients with T2DM have hepatic iron depositions and that their serum ferritin levels are significantly higher than those of normal subjects [26]. Neuroimaging studies have shown hippocampal iron deposits in patients with T2DM [6]. The present results show that T2DM rats treated with AAV-hepcidin had significantly reduced iron deposition in both the left and right hippocampus compared with M-blank rats, which is consistent with previous studies that AAV-hepcidin alleviated hippocampal iron deposition in rats [16]. These findings revealed that hippocampal iron deposition in T2DM rats is closely related to the expression of hepcidin. Hepcidin, a cysteine-rich antibacterial peptide synthesized and secreted by the liver, plays a negative role in regulating iron balance in the body [13], and its deficiency is responsible for iron overload in the body [27]. Not only have decreased hepatic hepcidin levels been observed in rats treated with STZ and HFD [25], but also patients with T2DM have iron deposition in the liver and decreased serum hepcidin levels [28]. The strategy of using a recombinant adeno-associated virus to target a speci-

Table 4. Results of the Morris water maze test for the three T2DM groups

Groups	M-hepcidin (n = 8)	M-blank (n = 8)	M-saline (n = 8)	*p* value	*p* value for post hoc analysis		
					a	b	c
Day 1 (s)	43.44±3.19	44.64±3.16	36.23±6.20	0.365	0.851	0.262	0.193
Day 2 (s)	34.12±3.60	39.80±6.85	32.62±4.52	0.593	0.446	0.84	0.338
Day 3 (s)	27.28±3.24	27.15±4.31	34.36±6.19	0.482	0.986	0.303	0.295
Day 4 (s)	25.64±4.1	33.56±6.13	32.67±6.96	0.586	0.35	0.406	0.916
Day 5 (s)	20.50±2.18	33.49±4.84	32.62±4.89	0.070	0.039*	0.052	0.884
Crossings	2.88±0.3	1.50±0.33	1.63±0.46	0.028*	0.015*	0.026*	0.813
Time in the target quadrant (s)	14.55±1.15	11.51±1.62	10.36±2.05	0.203	0.207	0.087	0.627

[a]Differences between the M-hepcidin and M-blank groups. [b]Differences between the M-hepcidin and M-saline groups. [c]Differences between the M-blank and M-saline groups. *, $P < 0.05$.

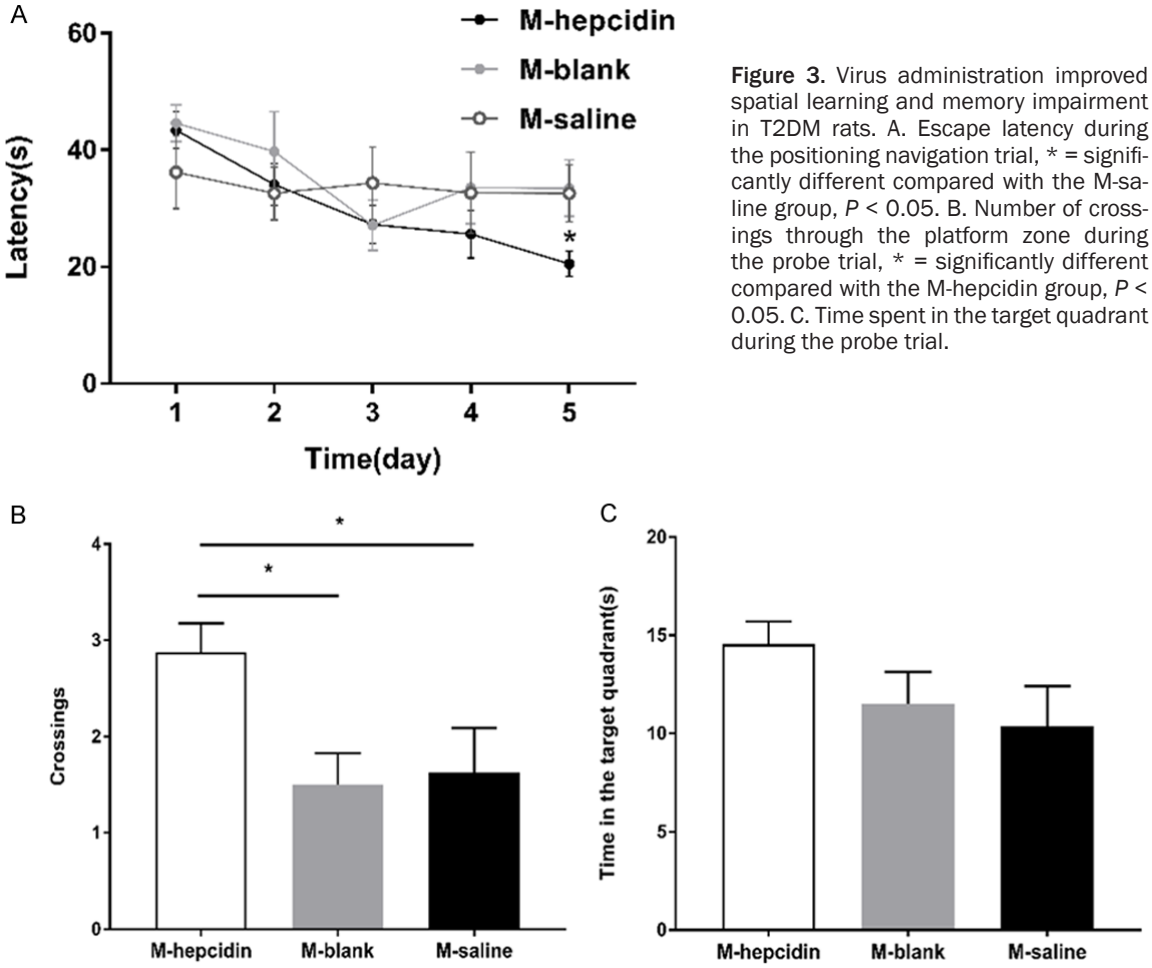

Figure 3. Virus administration improved spatial learning and memory impairment in T2DM rats. A. Escape latency during the positioning navigation trial, * = significantly different compared with the M-saline group, $P < 0.05$. B. Number of crossings through the platform zone during the probe trial, * = significantly different compared with the M-hepcidin group, $P < 0.05$. C. Time spent in the target quadrant during the probe trial.

fic gene sequence and increase that gene's expression has already been used in clinical treatment [29]. Recent studies have shown that AAV-hepcidin increased hepcidin expression and then reduced brain iron content in iron-overloaded rats by downregulating iron-transport proteins [16]. Therefore, the speculation was that insufficient hepcidin in T2DM leads to altered control of iron-transport proteins, and subsequent hippocampal iron deposition. The reduction in hippocampal iron content in the M-hepcidin group was therefore likely due to the AAV expression of hepcidin.

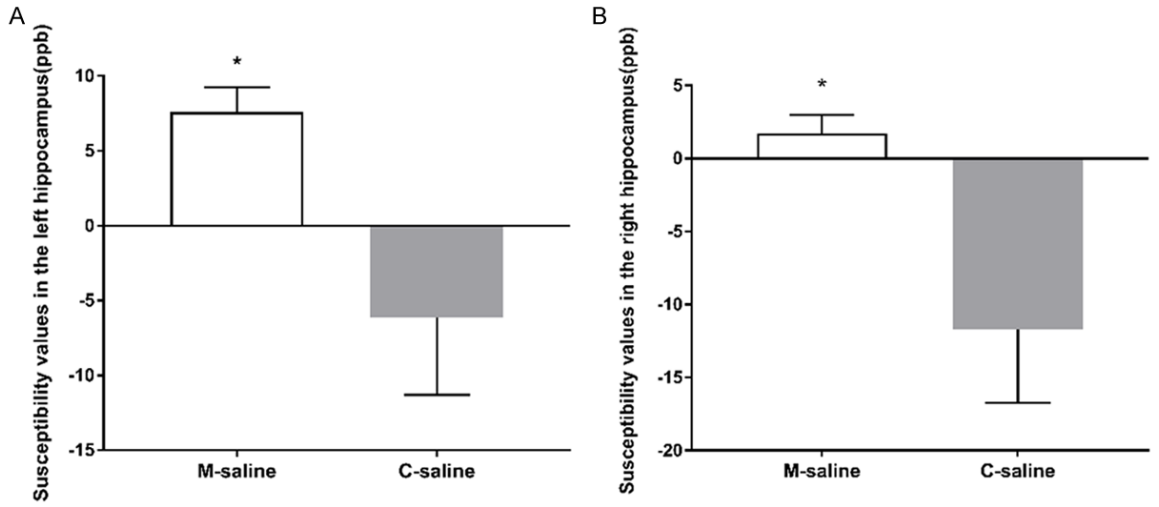

Figure 4. Hippocampal iron deposition in T2DM rats. A. Differences in susceptibility values in the left hippocampus. B. Differences in susceptibility values in the right hippocampus, * = significantly different from the C-saline group, $P < 0.05$.

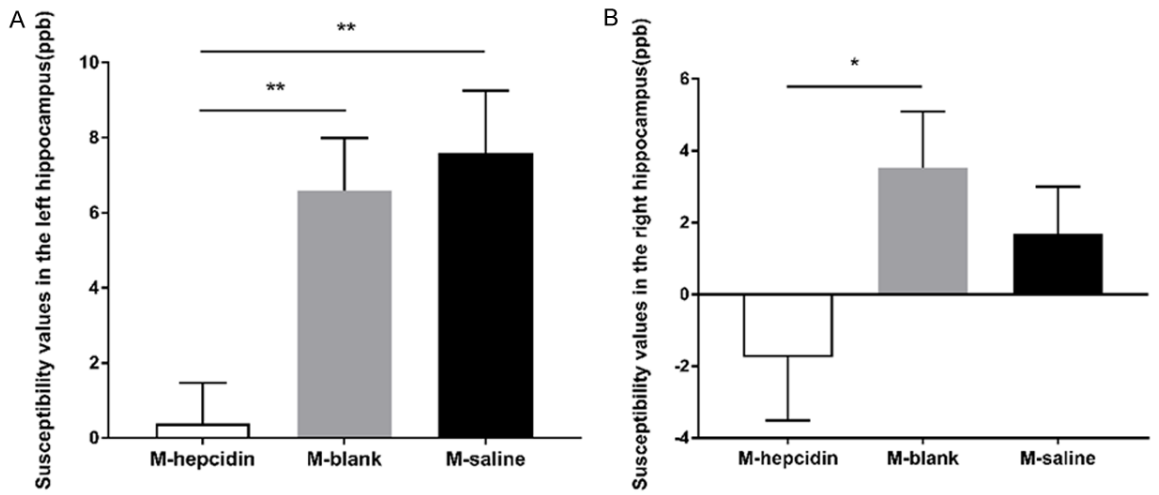

Figure 5. Virus administration reduced hippocampal iron deposition in T2DM rats. A. Differences in susceptibility values in the left hippocampus, ** = significantly different from the M-hepcidin group, $P < 0.01$. B. Differences in susceptibility values in the right hippocampus, * = significantly different from the M-hepcidin group, $P < 0.05$.

The present study has shown a significant difference in spatial learning and memory between T2DM rats and normal rats. T2DM rats had significantly worse spatial learning and memory compared with control rats, demonstrating that T2DM caused cognitive impairment, confirming other studies [3]. Similarly, MRI studies by Yang et al. have shown that patients with T2DM have hippocampal iron deposits that are negatively correlated with cognitive performance [6]. Iron is a crucial element for maintaining neural function, immune system function, muscle function, and energy metabolism [30]. However, iron deposits in the brain is the main cause of cognitive impairment [31] and neurodegenerative disorders [11], with a positive correlation shown between hippocampal iron deposition and cognitive impairment in patients with Alzheimer's disease [32]. Excess iron can also mediate oxidative stress and induce cell apoptosis [30]. It is well known that the hippocampus plays a prominent role in cognitive functions and is responsible for memory, including spatial memory [33], so iron

deposition in the hippocampus may damage cells through oxidative stress, leading to cognitive impairment [10]. Therefore, increases in hippocampal iron content in T2DM may be associated with cognitive function impairment. The present study has shown that rats in the M-hepcidin group performed better in spatial learning and memory compared with M-blank and M-saline rats indicates that AAV-hepcidin can improve cognitive impairment in T2DM rats. Gong et al. demonstrated that pretreatment with ad-hepcidin reduced iron content in the brain and prevented iron-induced oxidative stress in iron-overloaded rats [17]. Therefore, hippocampal iron overload in T2DM rats may have caused oxidative stress, leading to damaged hippocampal neurons and subsequent cognitive impairment. The present hepcidin therapy may have promoted the expression and release of hepcidin. As a result, hippocampal iron content in T2DM rats was likely decreased by the downregulation of iron-transport proteins, cell damage was averted by the inhibition of iron-mediated oxidative stress, and cognitive impairment was improved.

In conclusion, the present research demonstrated that T2DM rats had cognitive impairment and hippocampal iron deposits and that hepcidin therapy could decrease hippocampal iron overload and improve cognitive impairment in T2DM. These findings may help to reveal the role of hepcidin in iron homeostasis in T2DM, and provide a new basis for the diagnosis, treatment and monitoring of T2DM-associated cognitive impairment.

This study has some limitations. First, in addition to a relatively small sample size, the ROI was limited to the hippocampus, as this was the main area of our current research. A large-sample study, with multiple ROIs that include cerebral cortex and substantia nigra, should be conducted in the future. Second, although hippocampal iron deposition was detected by MRI, the analysis of iron content in the specimens and the correlations between QSM values and iron content in the specimens will be investigated in future studies. Third, there was no histopathological analysis of tissue in the present investigation, such as for the expression analysis of relevant marker genes. These analyses will be performed in future research.

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Disclosure of conflict of interest

None.

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