

Intracerebroventricular administrations of angiotensin IV (Ang IV) ameliorate cognitive disorder in diabetic rats

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Abstract

Cognitive impairment is a common complication of diabetes. Hippocampus plays an important role in cognitive function. In hyperglycemia, synaptophysin, a crucial synaptic vesicle membrane protein in hippocampus neuron is found to be down-regulated. Recent evidences have shown that angiotensin IV can facilitate memory acquisition and recovery. However, whether it can also improve cognitive functions of diabetic rats with cognitive disorder, and the possible mechanisms are uncertain. Hence, the objectives of this study. Forty five Sprague Dawley male rats were randomly divided into three groups: Control, diabetic group and diabetes with angiotensin IV treatment group. The cognitive functions, mainly learning and memory of the rats were evaluated using Morris water maze task. The synapses ultrastructure, relative mRNA concentrations and protein expression levels of synaptophysin in hippocampus CA1 area were estimated using transmission electron microscope, RT-PCR, immunohistochemistry and western blotting, respectively. Our study showed that in the diabetic rats with angiotensin IV treatment, the cognitive impairment as measured by Morris water maze task improved, the ultrastructure of synapses in hippocampus reversed, the relative mRNA concentrations and protein levels of synaptophysin in hippocampus significantly increased, when compared with diabetic rats. We conclude that angiotensin IV plays an important role in improving cognitive function of diabetic rats. The possible mechanisms are up-regulating the expression of synaptophysin and normalizing the ultrastructure of synapses in hippocampus.

INTRODUCTION

There are many evidences which suggest that hyperglycemia can damage the central nervous system and these diabetes-induced central nervous system complications is known as diabetic encephalopathy.¹⁻³ The predominant manifestations of diabetic encephalopathy involve deficits in cognitive function, failure of learning and memory.⁴⁻⁶ Recent research shows that angiotensin IV (Ang-IV), a six peptide fragment of angiotensin II, can facilitate memory acquisition and recovery, the binding site of which is mainly localized in hippocampus. Furthermore, its corresponding ligands AT4 receptor system, an insulin-regulated membrane aminopeptidase (IRAP), is recently confirmed to have effect on memory enhancement.⁷⁻¹¹

Synaptophysin, one of the most abundant polypeptide components of synaptic vesicles, is a constituent of neuronal cytoskeleton

proteins, the expression of which is decreased in hyperglycemia. It is believed that synaptophysin can modulate the synaptic vesicle cycle, and have a close relationship with synaptic plasticity.¹²⁻¹³ Renin-angiotensin system (RAS), other than being known for regulating blood pressure, sodium and water balance, also plays an important role in normal cognitive processing. It may have a role in cognitive dysfunction associated with diabetes mellitus. The aim of this study is to investigate the effect of Ang-IV on the expression of hippocampus synaptophysin and the behavior transformation of diabetic rats, aiming to determine the probable impact of Ang-IV on cognitive function.

METHODS

Animals

Sixty adult male normal Sprague Dawley (SD) rats (300–320g, approximately 10 weeks old)

provided by the Experimental Animal Center of Chongqing Medical University were the subjects in this study. They were group-housed with regular feeding with a regular 12-h light–dark cycle, and living under standard room condition (temperature: 25–28 °C, humidity: 60–65%) with a diurnal cycle. The experiments were performed according to internationally followed ethical standards and approved by the Research Ethics Committee of the Central Drug Research Institute and Committee for the Purpose of Control and Supervision of Experiments on Animals.

Experimental induction of diabetes

The 60 rats were divided into three groups randomly: normal rats (Control), diabetic rats with no Ang-IV treatment (DM), diabetic rats with Ang-IV treatment (DM+Ang-IV); each group consisting of 20 rats. The diabetic rats were successfully induced as described previously.¹⁴ Briefly, after abrosia for 12h, streptozotocin (STZ, Alexis Co., USA) were given, which were dissolved in 0.1 mmol/L citric acid-sodium citrate buffer, pH 4.2, at the dose of 60 mg/kg via conventional intraperitoneal injection (i.p.). Seventy two h later, those animals with fasting blood glucose levels over 16.7mmol/L (LifeScan glucometer: B7MD-BY, Johnson and Johnson Co., USA) were regarded as diabetic. After 12 weeks, the diabetic rats were induced again.¹⁵ Finally, there were 15 rats in each group in this study. Rats in DM+Ang-IV group were injected with Ang-IV (Phoenic pharmaceutical Co., LTD, USA), while DM group and Control were given equal volume of physiological saline solution. A rat in group DM died, due to poor tolerance to hyperglycemia, and was removed from the study.

Morris water maze task

Morris water maze test was used to assess cognitive function. The assessments were not blinded. Test device consisted of a circular pool (150 cm in diameter and 30 cm high) made up of gray plastic filled with water and was divided into four equal quadrants. An escape platform (10 cm in diameter and 15 cm high) was placed in one of the four maze quadrants labeled A–B–C–D, submerged 1.0 cm below the water surface; with a water maze record analysis system. All the rats were trained continuously for 4 days. On the fifth day, the rats were subjected to *finding the submerged escape platform trials* where the time it takes to reach the submerged escape platform (escape latency) was determined as a

measure of the learning abilities. On the sixth day, *spatial exploring trials* were performed with the submerged escape platform removed, the rats' swimming trajectory and the number of times of crossing the site of original platform determined as a measure of the memory function.

Surgery

After anesthesia was induced (with chloralhydrate, 10mL/kg), the rat head was fixed on the brain stereotaxic device (Stoelting, USA) for positioning of the lateral ventricle. As described previously¹⁶, a hole in the skull was drilled. A stainless steel cannula with 0.9 mm external diameter and 0.5 mm inner diameter was inserted into the lateral ventricle vertically following the drilled hole. At insertion of about 3.5 mm depth, cerebrospinal fluid would flow out when the inner tube was pulled out slowly, which was regarded as successful surgery. Four days after the surgery, the lateral ventricle micro-injection was carried out. Five uL Ang-IV (1nmol/uL) diluted with physiological saline was infused slowly into the lateral ventricle by a micro syringe. The whole process was performed in a constant speed. Ang-IV solution was injected one time a day for a week per rat in group DM+ Ang-IV, while the rats in group Control and group DM were given equal volume physiological saline solution.

Ultramicrostructure observation

After the ethology tests were completed, the rats were anesthetized with 10% chloral hydrate (350mg/kg, i.p.), the brain was quickly removed, and the entire hippocampus was carefully dissected. The left hippocampus was used for reverse transcription-polymerase chain reaction (RT-PCR) and ultramicrostructure observation; the right hippocampus was used for western blotting and immunohistochemistry examination. According to the standard methods of transmission electron microscope (TEM), electron stains of hippocampus tissues with uranyl acetate and folic acid were carried out, and the synapse ultrastructure were observed by TEM (Hitachi-7500, Japan). We calculated the width of synaptic cleft through the measurements multiply amplification. Three electron microscopic sections were taken with each rat's hippocampus tissue. Five electron microscope visions of each electron microscopic sections were observed. The mean \pm standard deviation value was determined.

Immunohistochemistry

Hippocampus tissues were embedded in paraffin, and were cut serially into coronal slice, and the expressions of synaptophysin were determined according to the kit's instructions (Wuhan Boster Co., LTD, China). Beijing University of Aeronautics and Astronautics Medical Image Analysis Management System was used to perform the image statistic analysis. Six pieces of slices per animal were chosen randomly, and 6 visions per slice were analyzed randomly with microscope. The average optical density was then determined.

RT-PCR

Total RNA was extracted with TRIzol reagent (Life Technologies, Rockville, MD, USA) following the manual instructions, and mRNA was purified with an Oligotex mRNA kit (Qiagen, Hilden, Germany). Primer sequences were 5'-CAGCCGTGTTTCGCTTTCAT-3', and 5'-CCACCCGTGGGATCTTCAT-3'. Five μ l PCR products were used for 1% agarose gels electrophoresis to authenticate and analyse, and they were then recorded with photograph. Digital gel image analysis system was used to scan the amplified specific bands; and the bands were analyzed and assayed by Quantity One software. The relative quantities (or relative concentration values) were calculated as synaptophysin/ β -actin.

Western blotting

The homogenate was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was stored at 4°C. Total protein concentrations were determined with a UV spectrophotometer using a modified Bradford assay (Beckman Coulter, Fullerton, USA). Equal amounts of protein from each sample (40 μ g) were mixed with 15 μ l sample buffer. Samples were separated by electrophoresis on 8% polyacrylamide gels. Separated proteins were

transferred onto nitrocellulose (NC) membrane at 30 V for 12 h. The membrane was blocked with 5% dried, defatted milk in TBST buffer for 1 h at room temperature. Blots were probed with specific rabbit anti-rats synaptophysin polyclonal antibodies (1:200, Lab Vision Corporation, USA). After washing with TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit antibodies (1:2500, Zhongshan Biotechnology, China). The optical densities of the specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, China).

Statistical analysis

Results were expressed as mean \pm standard deviation and were analysed by SPSS17.0, and homogeneity of variance among groups were tested. The data obtained were analyzed by one-way analysis of variance (ANOVA), followed by Student-t test. Statistical significance was defined as $p < 0.05$.

RESULTS

Morris water maze

Table 1 lists the results of Morris water maze test. For the *finding the submerged escape platform trials*, when compared with Control, the DM group showed significantly prolonged escape latency (74.98 ± 6.03 s, $p < 0.01$). The escape latency of DM+Ang-IV group (52.44 ± 7.54 s) was shortened when compared with DM group ($p < 0.05$), while prolonged slightly when compared with Control (42.7 ± 5.93 s), but the later was not statistically significant.

For the *spatial exploring trials*, the DM group crossed the site of original platform significantly less number of times as compared to the Control (1.48 ± 0.27 vs 2.62 ± 0.22 , $P < 0.01$). This was reversed in the DM+Ang-IV group, with

Table 1: The result of spatial exploring trials and finding the submerged escape trials of Morris water maze study

Group	No. of rats	No. of times crossing the site of original platform	Escape latency in seconds
Control	15	2.62 ± 0.22	42.7 ± 5.93
DM	14	1.48 ± 0.27	74.98 ± 6.03
DM+Ang IV	15	2.31 ± 0.28	52.44 ± 7.54

DM, diabetes rats with no Ang-IV treatment; DM+Ang IV, diabetic rats with Ang-IV treatment

Table 2: The electron microscopic changes of the study subjects (mean±standard deviation)

Group	Rats number	Synaptic cleft (nm)	Synapse number
Control	15	20.1±1.76	3.2±0.40
DM	14	8.23±0.62	1.6±0.25
DM+Ang IV	15	16.45±1.01	2.7±0.32

DM, diabetes rats with no Ang-IV treatment; DM+Ang IV, diabetic rats with Ang-IV treatment

significantly more number of time crossing the site of original platform when compared to the DM group (2.31 ± 0.28 vs 1.48 ± 0.27 , $P<0.05$). There was no significant difference in the number of times crossing the site of original platform when comparing the DM+Ang-IV group with the Control. (Table 1)

Transmission electron microscope

By electronic microscope, the structures of presynaptic membrane, postsynaptic membrane, and synaptic cleft in the hippocampus of Control were easy to determine. The postsynaptic membrane was a tad thicker than the presynaptic membrane, the synaptic vesicles were abundant, and cell organelles were copious in the body of the neuron. In the DM group, the synapses showed pervasive changes with the axoplasm of presynaptic membrane and the axoplasm of postsynaptic membrane nearly blending together. The structures were ambiguous, and part of synapses could not be distinguished between the presynaptic membrane and postsynaptic membrane. Their synaptic cleft became narrow or even disappeared when compared to Control (8.23 ± 0.62 nm vs 20.1 ± 1.76 nm, $P<0.005$). The numbers of synaptic vesicle were also significantly reduced when compared to Control (1.6 ± 0.25 vs 3.2 ± 0.40 , $P<0.005$), with the mitochondrial cristae became short and the swollen. After intervention,

the ultrastructure of DM+Ang-IV group gradually normalized, with the synaptic cleft (16.45 ± 1.01 nm) and the synapse number (2.7 ± 0.32) not significantly different from the Control. (Table 2, Figure 1).

Immunohistochemical assay

When compared to Control, the mean optical density of positive synaptophysin neurons in hippocampus were less in the DM group (0.151 ± 0.021 vs 0.223 ± 0.016 , $P<0.01$). After intervention, the expression of synaptophysin increased in the DM+Ang-IV group when compared to the DM group (0.206 ± 0.019 , $P<0.05$). There was no significant difference between the DM+Ang-IV group and Control. (Table 3, Figure 2)

RT-PCR analysis

As the immunohistochemical assay results had demonstrated the differential expression of synaptophysin, to determinine if altered expression of synaptophysin mRNA was involved, the RT-PCR were carried out and relative concentration (RC) of synaptophysin/ β -actin was determined. The RC values of three groups were showed as mean \pm standard deviation, and statistical analysis performed (Figure 3).

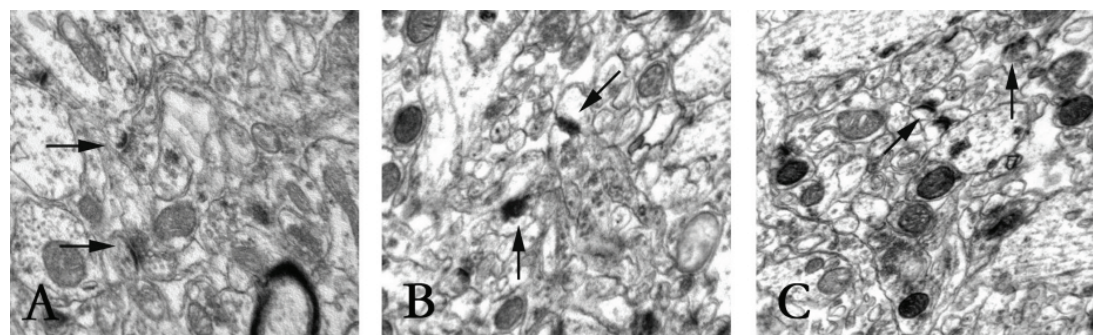


Figure 1. The results of synaptic ultrastructure in hippocampus of each group (TEM, $\times 20000$). The arrows show the synapses. (A) Synaptic structure in CA₁ region of the hippocampus by electronic microscope in Control; (B) DM group; (C) DM+Ang-IV group.

Table 3: The expression of synaptophysin in rat hippocampus (mean±standard deviation)

Group	Rat number	The mean optical density
Control	15	0.223±0.016
DM	14	0.151±0.021
DM+Ang-IV	15	0.206±0.019

DM, diabetes rats with no Ang-IV treatment; DM+Ang IV, diabetic rats with Ang-IV treatment

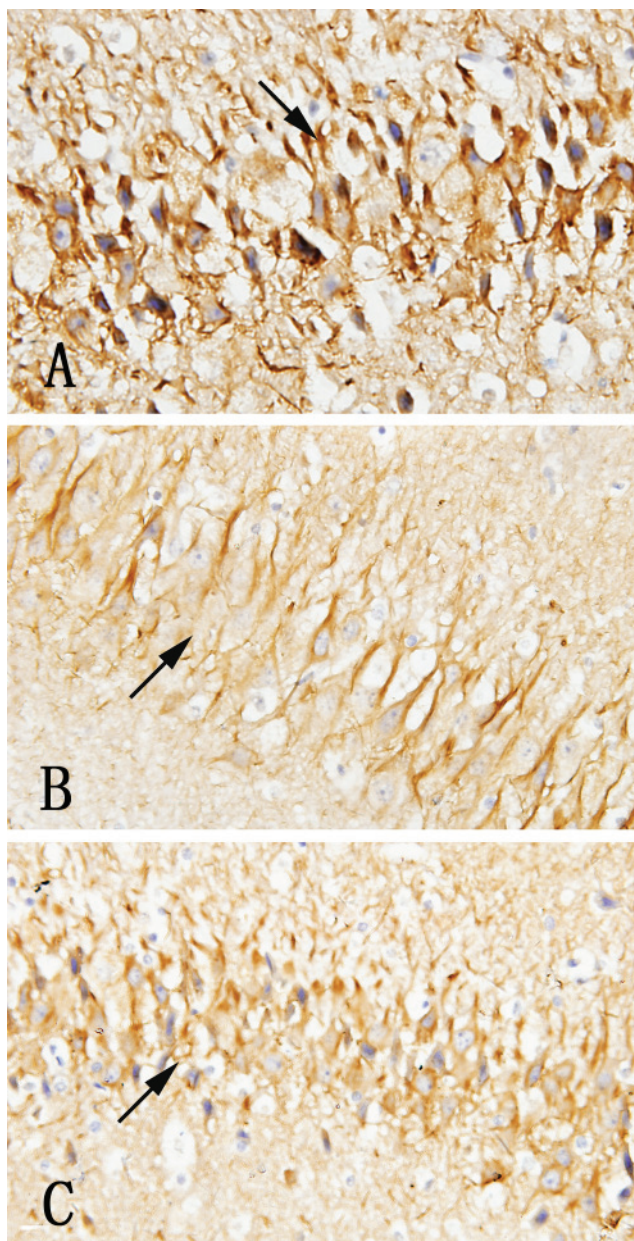


Figure 2. Expression of synaptophysin in hippocampus of rats (staining methods of SABC in immunohistochemical method, ×400). (A) The positive synaptophysin neurons in hippocampus appear tan, as shown by the arrows in Control; (B). DM group. (C). DM+Ang-IV group. Their mean optical density values were shown in Table 1.

Western blot assay

Western blot was also carried out for quantitative determination of protein. The relative optical density values of synaptophysin/ β -actin were determined and statistically analysed. (Figure 4).

DISCUSSION

Previous studies have shown that hippocampus plays a vital role in the processing of stored information.¹⁷ In the four subregions of hippocampus, CA1 is closely associated with learning and memory.¹⁸ Synaptophysin as a functional presynaptic membrane protein participates in multiple physiological activities associated with learning and memory in the hippocampus neurons.¹⁹ In this study, the synapse ultrastructure of the hippocampus CA1 region of diabetic rats showed various pervasive changes. The axoplasm of presynaptic and postsynaptic membranes were blending together; the synaptic cleft became verengt or even disappeared; and the number of synaptic vesicle was significantly reduced. These changes of structures probably resulted in impairment of the physiological function of the synapses.

Morris Water Maze is the most popular method to test the rat's cognitive function, by making the rat learns to find the submerged escape platform under the water. Their memory ability in spatial topesthesia and sense of direction (space orientation) were measured by estimating the number of times the rats crossing the site of original platform and the trajectory chosen. In this study, the result of Morris water maze suggested that the diabetic rats had cognitive dysfunction. When compared to the control, the DM group took significantly longer escape latency to find the platform, and had less number of times crossing the site of original platform. As we have also shown the changes in the synapse ultrastructure of the hippocampus, it can be inferred that the damage to the synapse is an important factors leading to cognitive impairment of diabetic rats.

Previous studies have reported a close association between renin-angiotensin system (RAS) and cognitive function. Ang-IV, a recently discovered metabolite of Angiotensi II, has a novel impact on learning and memory ability. For example, Olson *et al* reported that pharmacological doses of Ang-IV injected into the lateral ventricle could normalize the damaged spatial learning ability in the aged rat dementia model induced by acetylcholine receptor antagonist.²⁰ In our Morris water maze task study, the escape latency of the

DM+Ang-IV group was significantly shortened, and had significantly less time crossing the site of original platform when compared to the DM group. These results confirmed that Ang-IV can reverse the cognitive dysfunctions of diabetic rats.

As mentioned, synaptophysin is a glycoprotein located in presynaptic vesicles, and a distinctive marker of synaptic terminal, participating in the membrane fusion between synapse vesicles and presynaptic membrane, as well as the release of neurotransmitters. Therefore, synaptophysin changes of hippocampus can be associated with learning and memory abnormality. In the present study, the TEM study, immunohistochemistry and western bolt assay all showed the synaptophysin expression levels were significantly reduced in DM group as compared with Control. The synaptophysin expression levels were significantly up-regulated after the Ang-IV treatment, to a level close to the Control. It could thus be inferred that the effect of Ang-IV on improving the learning and memory ability was associated with the up-regulation of synaptophysin expression.

However, angiotensin appears to have other effects on the brain. Pelisch *et al.* reported that candesartan could improve the cognitive function of diabetic mice through blocking the angiotensin receptor type 1.²¹ Pavlatou *et al.* reported that candesartan could reset the hypothalamic-pituitary-adrenal axis of patients with type 2 diabetes, and improves their affect, which may indirectly affect the cognitive function.²² However, our studies involved intracerebroventricular (i.c.v.) administration of angiotensin IV rather than by peripheral routes, probably involving different binding site. De Bundel *et al.* also found i.c.v. administration of angiotensin IV to improve memory deficits in rats, implicating the involvement of AT1 receptors.²³

In conclusion, Ang-IV treatment can ameliorate cognitive dysfunction, and the possible mechanisms are up-regulating the expression of synaptophysin and normalizing the ultrastructure of synapses in hippocampus.

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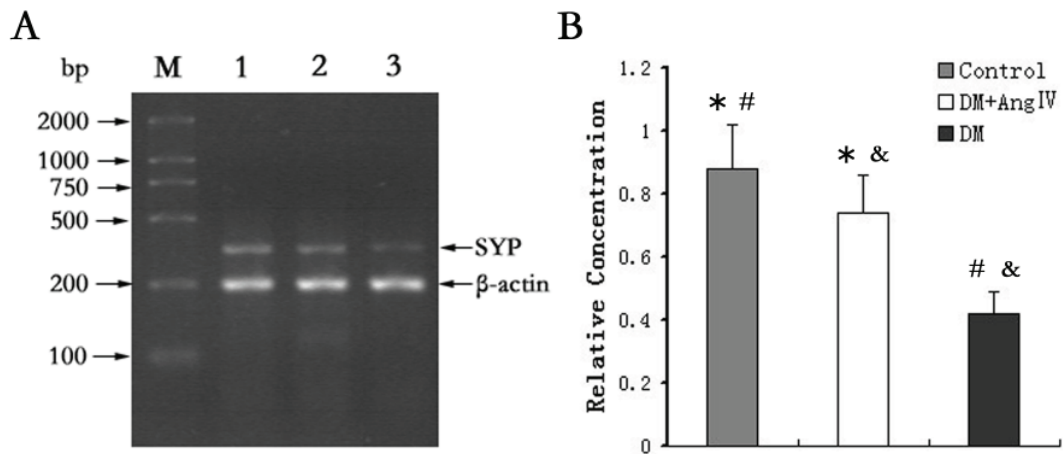


Figure 3. RT-PCR analysis of the mRNA levels of synaptophysin. In the left picture (A), Lane M: 2 kb plus DNA ladder; lane 1: Control; lane 2: DM+Ang-IV group; lane 3: DM group. Right histogram shows the relative concentrations of the three groups (B). The mRNA levels of group DM were reduced when compared with Control ($P < 0.01$) and DM+Ang-IV group ($P < 0.05$); while the difference between Control and DM+Ang-IV group was not significant.

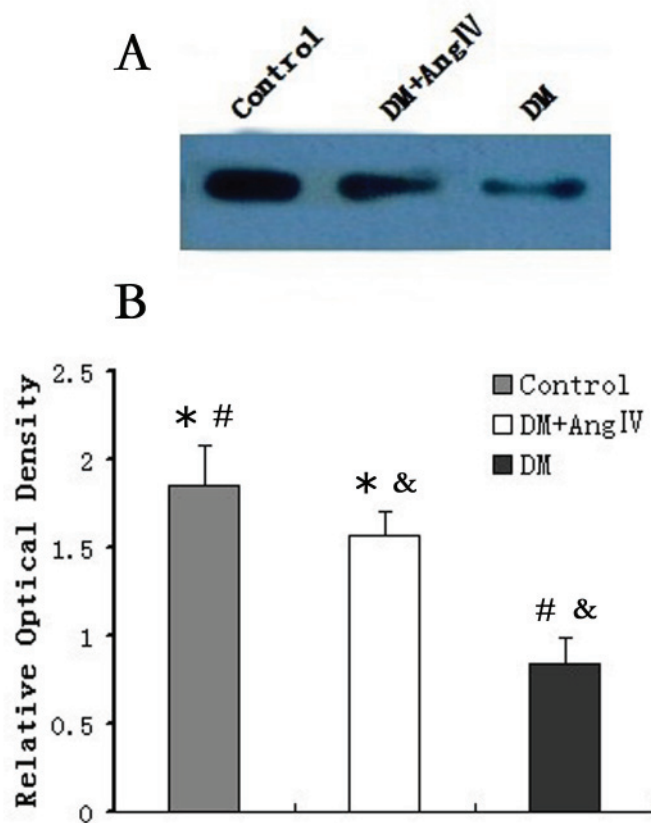


Figure 4. Analysis of the effect of Ang-IV on the protein levels of synaptophysin. (A). A typical western bolt assay images of the various study groups. (B). Statistical analysis of the synaptophysin expression levels. The synaptophysin expression levels of DM group were reduced when compared with Control ($P < 0.01$). There was no significant difference in the protein levels between Control and DM+Ang-IV group. The synaptophysin quantities of DM+Ang-IV group were greater than that of DM group ($P < 0.05$).

DISCLOSURE

Conflict of interest: None

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