MATERIALS AND METHODS

1. Animals

All experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committees in compliance with the NIH guidelines. Forty-eight male Wistar Rats weighing 180 to 200g from the National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand were used for this study. All animals were maintained under environmentally controlled conditions (25 ± 0.5 °C, 12 h light/12 h dark cycle). One week after arrival, the rats were divided into two groups (n = 24/group): normal diet group (ND) and a high fat diet group (HFD). Animals in the ND group were fed with standard laboratory chow (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand), which had an energy content of 4.02 kcal/g and 19.77% fat of total energy (%E) from fat for 12 weeks, whereas animals in the HFD group were fed by HFD which had an energy content of 5.35 kcal/g and 59.28% fat of total energy (%E) from lard. All animals were given free access to the diet and drinking water. At the 13^{th} week, the locomotor activities and the spatial learning and memory behaviors in all rats were determined using the open field test and the Morris Water Maze test, respectively. After that, rats in each group were divided into two subgroups (n=12/subgroup) to receive either vehicle (normal saline solution; 2 ml/kg/BW/day) or metformin (Glucophage, Merck Serono, Thailand; 15 mg/kg/BW twice daily), via gavage feeding for 21 days. The dose of metformin treatment in the present study was chosen for the clinically therapeutic range of 30 mg/kg/BW/day for T2DM (35), and the 21-day metformin treatment has been shown previously to improve peripheral insulin sensitivity in Zucker rats (36). At the end of the experimental period, the spatial learning and memory behaviors were determined

using the Morris Water Maze test. After the behavioral testing, animals were deeply anesthetized with isoflurane and sacrificed by decapitation after fasting for at least five hours. Their plasma was collected for further analysis. The brain was rapidly removed for measuring brain mitochondrial function and brain oxidative stress levels.

2. Plasma analysis

Plasma glucose and cholesterol levels were determined by the colorimetric assay (Biotech, Bangkok, Thailand). Plasma insulin levels were determined by Sandwich ELISA kit (LINCO Research, MO, USA). Peripheral insulin resistance was assessed by Homeostasis Model Assessment (HOMA) (37; 38). Plasma malondialdehyde (MDA) was determined using high-performance liquid chromatography (HPLC)-based assay (39).

3. Open- field test

The open-field was used to detect locomotor activity and was modified from the method of Arakawa (40). The open field was made of black platform and consisted of a floor (75 cm ×75 cm) with 40-cm walls. The box floor was painted with white line (6 mm) to form 25 equal squares. During a two minute observation period, the rat was placed at one corner of the apparatus facing the wall. The number of total lines crossed was recorded for 120 seconds.

4. Morris water maze test

The Morris Water Maze (MWM) test was modified from the method of Vorhees and Williams (41) for learning and memory behavior assessment. Learning and memory behavior evaluations were performed in a 170-cm diameter water pool and virtually divided into four quadrants. (Figure 2) The pool was filled with water (26±1◦C) and was made opaque with wheat. A clear platform of ten centimeters in diameter was submerged approximately one cm beneath the water surface, located

in a designated target quadrant. (Figure 3) The MWM test was done before and after the treatment in each rat. Each test included two different assessments; acquisition test (existed platform) and probe test (non-existed platform). The acquisition test was performed for five consecutive days of training with four trials per day. Animals were given 120 seconds to locate the hidden platform, and any animals that did not find the platform within 120-second period were guided to the platform. After the platform was found, the animal was allowed to remain on the platform for 15 seconds before the next test was done by placing the animal into the other three remaining quadrants as a starting point. The acquisition time was recorded at the time the animal got into the water and ending at the time the animal reached the submerge platform. In the probe test, the animals were tested on the sixth day with only one starting point. The probe time was the time the animals spent in the target quadrant.

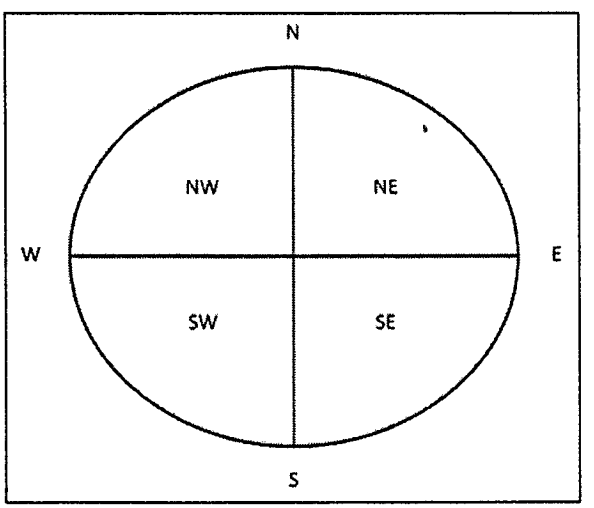

Figure 2: Four cardinal points and four quadrants of water pool

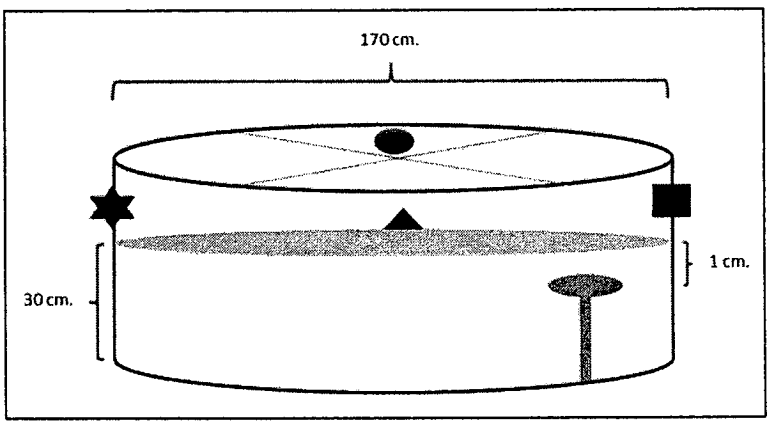

Figure 3: The water pool set up

5. Preparation of brain mitochondria

Brain mitochondria were isolated as described in our previous study (31). Briefly after decapitation, the brain was removed and put into five ml of ice-cold MSE solution, transferred to ten ml of ice-cold MSE-nagarse solution (0.05% nagarse in MSE solution), and homogenized at 600 rpm/min using a homogenizer. Then the brain homogenate was centrifuged at 2,000×g for four minutes and the supernatant was collected and further centrifuged at 12,000×g for nine minutes. Mitochondrial pellets were collected, resuspended in ten ml of ice-cold MSE-digitonin solution (0.02% digitonin in MSE solution) and further centrifuged at 12,000×g for eleven minutes. Finally, the mitochondrial pellets were resuspended with respiration buffer (150 mM KCl, 5 mM HEPES, 5 mM $K_2HPO_4.3H_2O$, 2 mM L-glutamate, 5 mM pyruvate sodium salt). The mitochondrial protein was measured by the BCA assay (42).

5.1 Brain mitochondrial reactive oxygen species (ROS) assay

Brain mitochondrial reactive oxygen species (ROS) was measured by dichloro-hydrofluoresceindiacetate (DCFHDA). The protein of the brain mitochondria (0.4 mg/ml) was stained with 2-μM DCFHDA at 25°C for 20 minutes, and evaluated

by a fluorescent microplate reader at the excitation wavelength of 485 nm and emission wavelength at 530 nm. The fluorescence was determined using a fluorescent microplate reader (31; 42).

5.2 Brain mitochondrial membrane potential ($\Delta\Psi m$) assay

The mitochondrial membrane potential ($\Delta\Psi m$) in isolated brain mitochondria was measured by fluorescent dye 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl benzimidazolcarbocyanine iodide (JC-1). JC-1 monomer form (green) fluorescent was excited at 485-nm wavelength and detected at the emission wavelength of 590 nm, and JC-1 aggregate form (red) fluorescent was excited at 485-nm wavelength, and detected at the emission wavelength of 530 nm. The brain mitochondrial protein (0.4 mg/ml) was stained with JC-1 dye at 37 °C for 15 minutes. The mitochondrial membrane potential was determined as fluorescence intensity by using a fluorescent microplate reader. The change in mitochondrial membrane potential was calculated as the ratio of red to green fluorescence intensity (31; 42).

5.3 Brain mitochondria swelling assay

The brain mitochondrial swelling was determined by measuring the change in the absorbance of the brain mitochondrial suspension. The brain mitochondrial protein (0.4 mg/ml) was incubated in a two ml respiration buffer. The suspension was read at 540 nm by using a microplate reader. The mitochondrial swelling was indicated by a decrease in the absorbance (31; 42).

6. Plasma and brain malondialdehyde (MDA) levels determination

A high performance liquid chromatography (HPLC) method was used to evaluate the plasma and brain melondialdehyde (MDA) concentration as an indicator of oxidative stress (43). Briefly, the brain was homogenized in phosphate buffer pH 2.8. The plasma and brain homogenate were mixed with 10% trichloroacetic acid (TCA)

containing butylated hydroxytoluene (BHT), incubated at 90 °C for 30 minutes, and centrifuged at 6,000 rpm for 10 minutes. The supernatant was mixed with H_3PO_4 and thiobabituric acid solution (TBA), incubated at 90°C for 30 minutes to produce thiobarbituric acid reactive substances (TBARS). MDA levels were measured by the HPLC system, and was determined directly from the standard curve, and reported as the MDA equivalent concentration.

Statistical analysis

Data were expressed as mean ± SE. The comparison was made between the two groups prior to the treatment using an independent *t*-test. Comparison among groups after metformin treatment was performed using one-way ANOVA followed by Fisher's least significant difference post hoc analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Metformin improved peripheral insulin resistance and oxidative stress levels in HFD-fed rats.

Twelve weeks of HFD consumption showed the characteristics of peripheral insulin resistance, such as increased body weight, visceral fat, plasma cholesterol, plasma insulin levels and HOMA index (as showed in Table 1). After 21 days of metformin administration, we found that plasma cholesterol, plasma insulin levels and HOMA index were significantly decreased in metformin-treated HFD rats (HFM), compared to the vehicle-treated HFD rats (HFV) ($p<0.05$; Table 1). Furthermore, we also investigated whether metformin can improve oxidative stress by measuring plasma and brain MDA levels. Our results demonstrated that plasma MDA and brain MDA levels were significantly increased following HFD consumption ($p<0.05$).

Metformin administration could significantly prevent the increment of plasma and brain MDA level in HFD rats ($p<0.05$). These findings indicate that metformin can improve both peripheral insulin resistance and oxidative stress in rats with HFD consumption.

Table 1. Effects of metformin administration on peripheral insulin sensitivity parameters and MDA levels in rats fed with normal or high-fat diet.

Parameters	**NDV**	**NDM**	**HFV**	**HFM**
Body weight (g)	443.33 ± 9.55	445.00 ± 14.55	561.67 ± 19.05*	558.33 ± 20.56*
Visceral fat (g)	29.07 ± 3.74	23.13 ± 3.40	47.30 ± 4.30*	44.75 ± 4.31*
Cholesterol	90.80 ± 4.33	89.30 ± 11.02	136.02 ± 7.36*	108.57 ± 5.84#
Plasma glucose (mg/dl)	154.65 ± 12.48	136.81 ± 19.61	172.7 ± 7.96	154.31 ± 8.73
Plasma insulin (ng/ml)	2.42 ± 0.31	2.18 ± 0.25	4.08 ± 0.47*	2.41 ± 0.45#
HOMA index	21.63 ± 2.81	18.10 ± 3.91	41.51 ± 5.25*	21.92 ± 4.03#
Plasma MDA (mmol/ml)	2.52 ± 0.15	2.63 ± 0.19	7.13 ± 0.13*	6.43 ± 0.20*,#
Brain MDA (mmol/ml)	1.46 ± 0.23	1.36 ± 0.28	2.30 ± 0.32*	1.55 ± 0.17#

*$p<0.05$ vs. NDV and #$p<0.05$ vs. HFV (NDV: normal diet rats with vehicle treatment, NDM: normal diet rats with metformin treatment, HFV: high fat diet rats with vehicle treatment and HFM: high-fat diet rats with metformin treatment)

Metformin improved brain mitochondrial function in HFD-fed rats.

To study the mechanism of metformin on brain mitochondria function, we determined whether the metformin improves ROS, membrane potentials and swelling in brain mitochondria. As shown in our previous study, 12-week of HFD consumption led to mitochondria dysfunction, such as increased mitochondria ROS, membrane potentials and swelling. In the present study, we further investigated whether metformin can improve mitochondria dysfunction caused by HFD consumption. We found that metformin treatment significantly decreased brain mitochondria reactive oxygen species (ROS) production ($p<0.05$; Figure 4A), brain mitochondrial membrane potential changes ($\Delta\Psi$) ($p<0.05$; Figure 4B) and absorbance values ($p<0.05$; Figure 4C) compared to HFV group. All of these findings suggest that metformin treatment has a beneficial effect on the improvement of mitochondria dysfunction caused by HFD consumption.

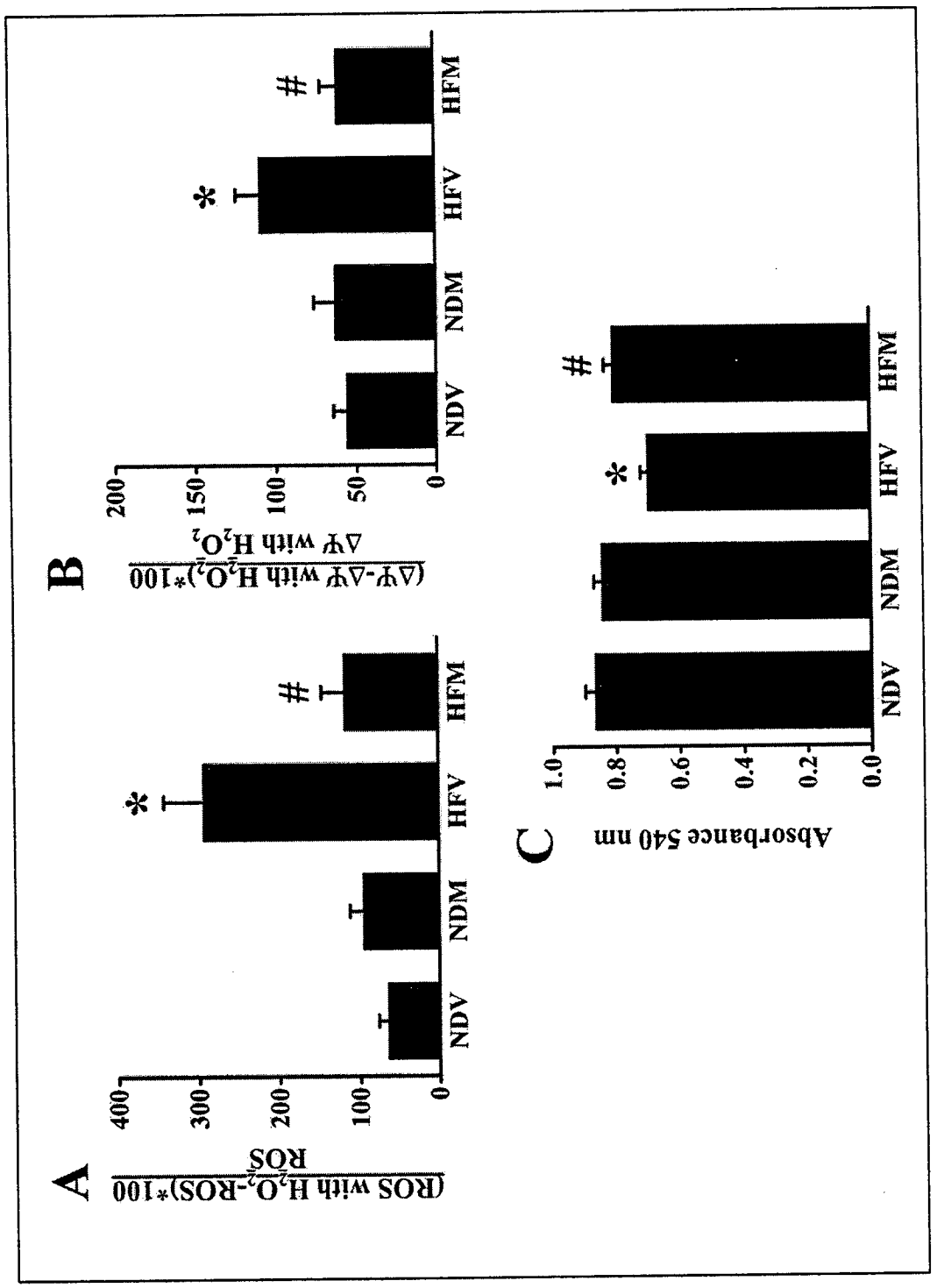

Figure 4: (A) Metformin administration reduced the production of ROS following H_2O_2 application caused by HFD consumption. (B) Metformin significantly decreased neuronal mitochondrial membrane potential change following H_2O_2 application compared with HFV. (C) Metformin significantly increased absorbance at 5 minute implies that improved neuronal mitochondrial swelling. *p<0.05; NDV vs. HFV, #p<0.05; HFV vs. HFM; NDV: normal diet rats with vehicle treatment, NDM: normal diet rats with metformin treatment, HFV: high fat diet rats with vehicle treatment and HFM: high-fat diet rats with metformin treatment

Metformin improved learning and memory in HFD-fed rats measured by Morris water maze (MWM) test

In the present study, we used Morris water maze test to determine the effects of metformin on learning and memory behavior in insulin resistant rats caused by HFD consumption. Our results demonstrated before metformin treatment, the mean of time to reach the platform in the acquisition test significantly increased in HFD fed-rats compared with ND fed-rats ($p<0.05$; Figure 5A). In addition, the mean of time spent in target quadrant significantly decreased in HFD fed-rats compared with ND fed-rats in probe test ($p<0.05$; Figure 5B). These findings indicated that 12-weeks of HFD consumption impaired in the learning and memory. Furthermore, we also investigated whether metformin improved learning and memory deficit caused by HFD consumption. We found that the mean of time to reach the platform during acquisition test in HFM group significantly decreased compared with HFV group ($p<0.05$; Figure 6A). In probe test, the mean of time spent in target quadrant in HFM group also significantly increased compared with HFV group ($p<0.05$; Figure 6B). These findings suggest that metformin has a beneficial effect on improving the impairment of learning and memory behavior induced HFD consumption.

Locomotor activity was determined by an open-field test. We found that the number of lines that the rats crossed during the test was not significantly different between the ND group (41.63±3.38 times/min) and the HFD group (44.14±2.81 times/min). These findings indicate that the locomotor activity of these rats were not different between the two diet groups.

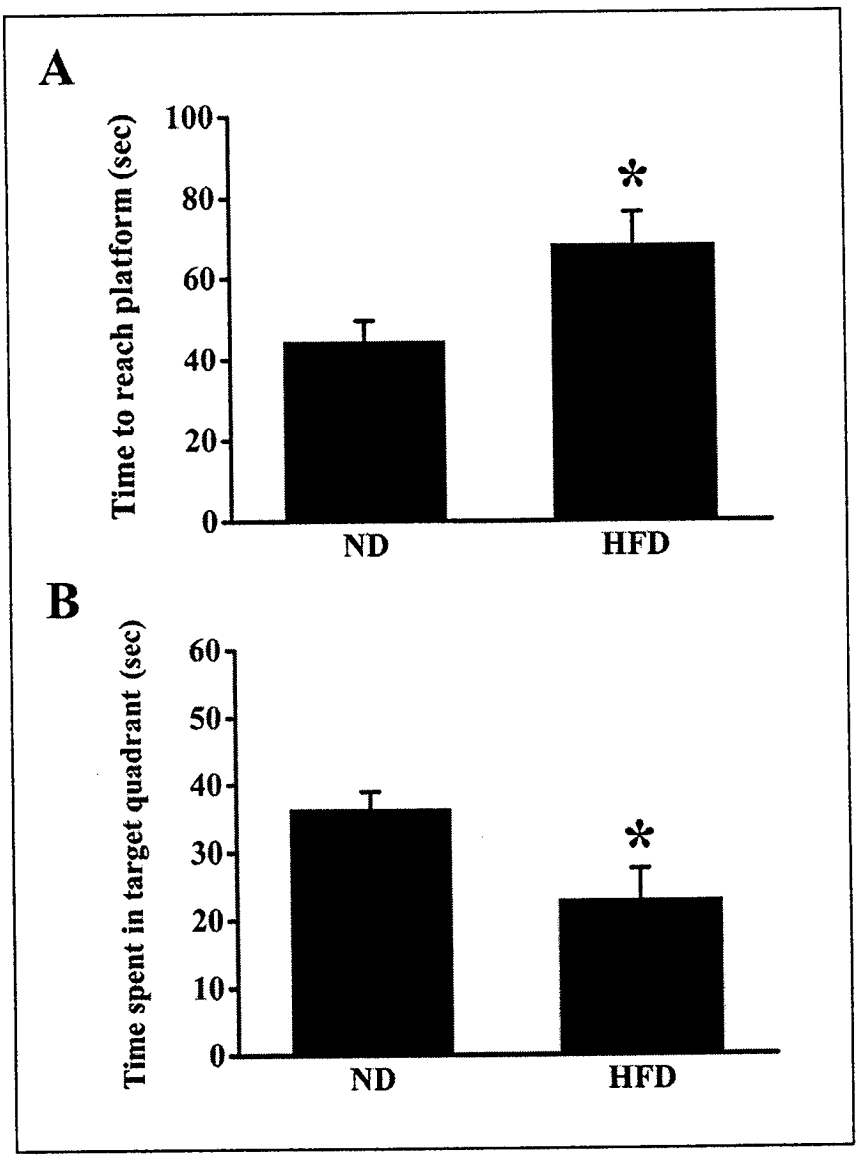

Figure 5: HFD consumption decreased learning and memory, which indicated by increased time to reach the platform in acquisition test (A) and decreased time spent in target quadrant in the probe test (B). *p<0.05; NDV vs. HFV, #p<0.05; HFV vs. HFM; NDV: normal diet rats with vehicle treatment, NDM: normal diet rats with metformin treatment, HFV: high fat diet rats with vehicle treatment and HFM: high-fat diet rats with metformin treatment

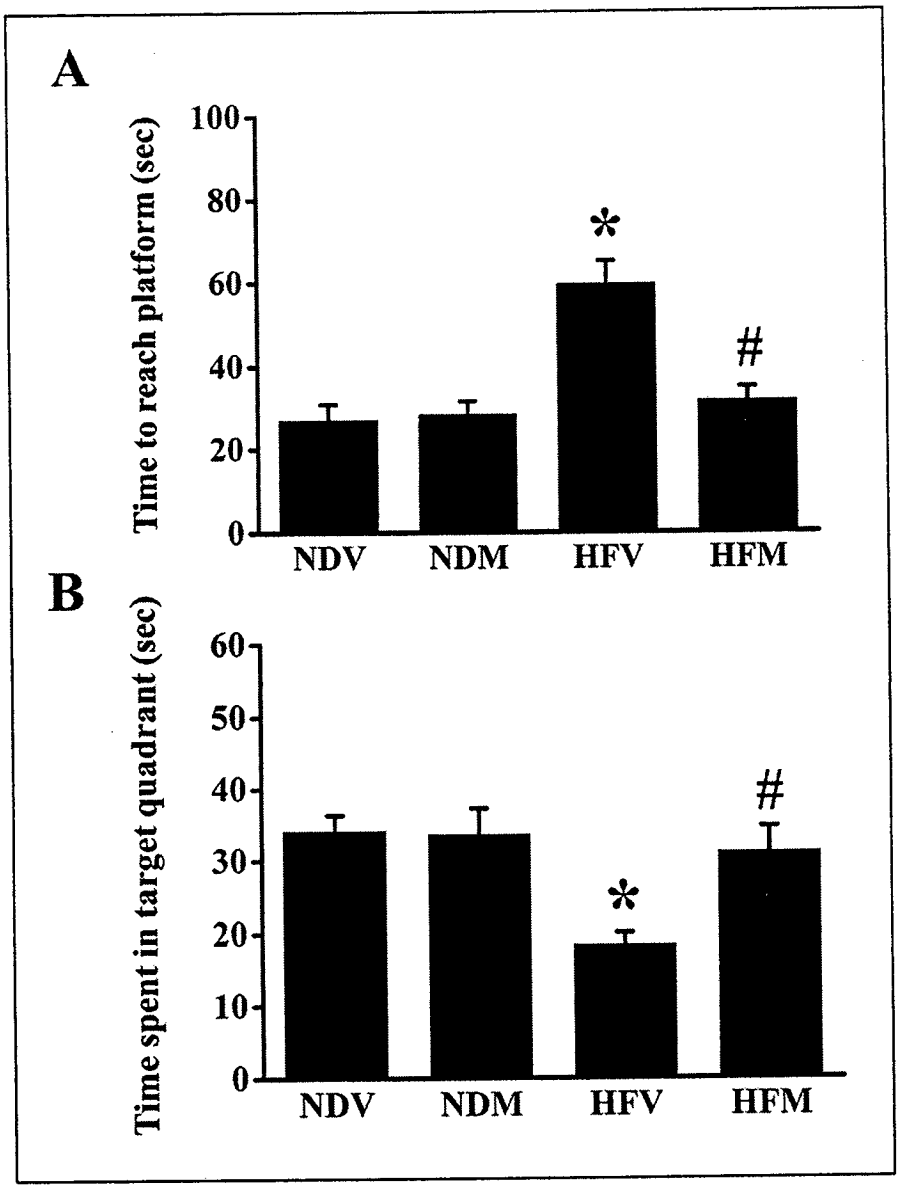

Figure 6: Metformin significantly improved learning and memory deficit, which indicated by decreased time to reach the platform in acquisition test (A) and increased time spent in target quadrant in the probe test (B). *$p<0.05$; NDV vs. HFV, #$p<0.05$; HFV vs. HFM; NDV: normal diet rats with vehicle treatment, NDM: normal diet rats with metformin treatment, HFV: high fat diet rats with vehicle treatment and HFM: high-fat diet rats with metformin treatment