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Analysis of Learning and Memory Ability in an Alzheimer's Disease Mouse Model using the Morris Water Maze

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August 06, 2019

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Dr. Baja:

I wish to re-submit the manuscript titled "Analysis of learning and memory ability in Alzheimer' Disease mouse model using Morris water maze." The manuscript ID is JoVE60055R1.

We thank you and the reviewers for your thoughtful suggestions and insights. The manuscript has benefited from these insightful suggestions. I look forward to working with you and the reviewers to move this manuscript closer to publication in the *JoVE*.

The manuscript has been rechecked and the necessary changes have been made in accordance with the reviewers' suggestions. The responses to all comments have been prepared and attached herewith below.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,
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TITLE:

Analysis of Learning and Memory Ability in an Alzheimer's Disease Mouse Model using the Morris Water Maze

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KEYWORDS:

Morris water maze, protocol, behavior, Alzheimer' Disease, mouse, manual acupuncture

SUMMARY:

Herein, a protocol to conduct the Morris water maze tests to evaluate the ability of learning and memory of Alzheimer's Disease model mice and to assess the effect of manual acupuncture for treating them is described.

ABSTRACT:

A Morris water maze (MWM) experiment forces experimental animals to swim and learn to find a platform hidden in the water. It is widely used in scientific research to assess the learning and memory of animals. Due to the extensive use of the MWM test, visual experimental protocols are essential for researchers. This manuscript uses the latest studies to introduce the protocol of the MWM test. Alzheimer' Disease (AD) is characterized by a progressive loss of memory and cognitive function. An alternative and complementary treatment used for AD is Manual

Acupuncture (MA). To assess the learning and memory ability of AD model mice, the MWM test was conducted. The visible platform trial, hidden platform trial, probe trial, and reversal trial of MWM were used to evaluate spatial learning and memory ability. In the visible platform trial, the swimming speed and escape latency of mice in different groups was not significantly different. In the hidden platform and reversal trials, the AD group showed a long escape latency. The escape latency decreased significantly after the MA treatment. Low platform crossover number and the proportion of time in the SW quadrant in the probe trial increased after the MA treatment ($p < 0.05$ or $p < 0.01$). The results of the MWM tests suggest that MA can effectively improve the spatial learning and memory abilities of AD model mice. Rigorous experimental operations provided assurance of the reliability of the results.

INTRODUCTION:

Currently, the MWM experiment has become the most widely used and the standard behavioral experiment to evaluate the spatial learning and memory of animals[1]. It was initially designed by the British psychologist Richard G. Morris and has constantly been improved. Many advantages such as minimal training, cross-species utility, insensitivity to differences in body weight, and repeated testing ability of MWM make it the best method for assessing cognitive function[2]. Alzheimer's disease (AD) is a major medical problem, primarily characterized by a decline in memory processing and cognitive function[3]. MWM is an indispensable experimental means to evaluate the learning and memory ability of AD model animals and the effectiveness of intervention methods. MWM experiments are generally time consuming (6–11 days) and involve many variable factors[4]. Although there are many articles about water maze experiments, in practice, researchers lack a coherent protocol. Therefore, an intuitive and rigorous protocol process video is particularly important. Using a previous experiment as an example[5], all the steps of the MWM are described. Using MWM, previous studies suggested that acupuncture could relieve the symptoms of AD model mice[5-7].

Herein, the MWM protocol used in a recent study[5] is described to provide a simple and visible method for researchers to assess the spatial learning and memory of AD model animals.

PROTOCOL:

This protocol was approved by the Animal Ethics Committee of Beijing University of Chinese Medicine, and it was in accordance with all guidelines for the Care and Use of Laboratory Animals of China. There was no accidental death situation during the experimental procedure, and no animals needed to be euthanized in this study.

1. Preparation

1.1. Purchase 30 male SAMP8 mice and 10 male SAMR1 mice (age: 8 months).

1.2. House the mice individually in individual ventilation cages at a temperature of 24 ±2 °C and a 12 h dark/light cycle.

1.3. Feed the mice with a standard pellet diet available ad libitum and provide sterile

drinking water.

1.4. Acclimate all the mice to the environment for 5 days before experimentation.

2. Grouping of animals

2.1. Randomly divide 30 SAMP8 mice into three groups (n = 10/group): the AD group, manual acupuncture (MA) group, and medicine (M) group.

2.2. Use 10 SAMR1 mice as the normal control (N) group[6].

3. Administration of donepezil hydrochloride tablets

3.1. Crush a donepezil hydrochloride tablet (5 mg/tablet) and dissolve it in 50 mL of distilled water.

3.2. Deliver the medicine prepared in step 3.1 at doses of 1 mg/kg to the mice using an oral gavage once a day[8] during the entire experiment, including the days when the MA treatment and MWM tests are performed.

4. Administration of manual acupuncture

4.1. Immobilize the mice of the MA group in mouse bags.

4.2. Use disposable sterile acupuncture needles (0.25 mm x 13 mm) and apply the flat thorn method of MA on Baihui (GV20) and Yintang (GV29)[5] toward the nose for 20 min. Ensure that the needle depth is 0.2–0.3 cm.

4.3. Bidirectionally twirl the manipulation within 90° at a speed of about 180 r/min every 5 min for ~15 s each time during the entire experiment, including days when MA treatment and MWM tests are performed.

5. MWM test

NOTE: At 24 h after the 15 consecutive days of treatment, subject the mice in the four groups to the MWM test. Conduct the visible platform trial, hidden platform trial, probe trial, and reversal trial in order.

5.1. Prepare for the MWM test.

5.1.1. Position MWM device and the signal acquisition and processing system in an experiment room designed to maintain sound insulation.

5.1.2. Put a circular white tank (diameter = 90 cm, height = 50 cm) surrounded by an opaque cloth in the middle of the MWM device.

5.1.3. Fix a video camera to the ceiling of the MWM device and connect it to a video recorder with an automated tracking system to collect the data.

5.1.4. Divide the water maze tank equally into four equal regions using two mutually perpendicular lines, labeled north (N), south (S), east (E), and west (W). Divide the pool area conceptually into four quadrants of the same size (NE, NW, SW, and SE).

5.1.5. Within the sight of the mouse, place visual cues of different shapes on the wall of each quadrant as visual references (e.g., squares, triangles, and circles).

NOTE: Distal cues are the animal's navigational reference points for locating the platform. Therefore, do not move them during the test. The position of the researcher is a potential distal cue and can influence the MWM. Therefore, the researcher should stay out of the sight of the mice while waiting for the animal to perform the test.

5.1.6. Fill the circular tank with water to a depth of 30 cm and maintain at 22 ± 2 °C with an electric heater.

5.1.7. Render the water opaque with about 150 g of milk powder.

5.2. Perform the visible platform trial.

5.2.1. Place a plastic circular platform (diameter = 9.5 cm; height = 28 cm) 1 cm above the water surface in any quadrant at random.

5.2.2. Put a black flag on the platform.

5.2.3. Release each mouse gently into the water at water-level from one of the four start locations facing the tank wall. Do not drop the mouse into the water.

5.2.4. Activate the computer tracking program as soon as the mouse is released into the water.

5.2.5. Give each mouse 60 s to search for the platform. At the end of each trial, place each mouse on the platform and allow it to stay on it for 10–30 s.

5.2.6. Observe the swimming trajectories of the mice on the computer, record the time the mouse took to find the platform as escape latency, and analyze the swimming speed.

5.2.7. Dry each mouse with towels and warm it with an electric heater.

NOTE: Place each mouse into the pool at each of the four different starting quadrants for four trials, moving the platform to a different location with each subsequent trial. The interval between two trials using each mouse is 15–20 min.

5.3. Perform the hidden platform trial/place navigation test.

5.3.1. Place the same platform without a flag in the SE quadrant.

5.3.2. Randomly place the mouse into the pool from each of the four quadrants (NE, NW, SW, N) facing the pool wall for four trials. Use a time interval of 15–20 min between two trials.

5.3.3. Give each mouse 60 s to search for the hidden platform.

5.3.4. Record the escape latency of each trial after the mouse climbs up to the platform for subsequent analysis.

5.3.5. Dry each mouse with towels and warm it with an electric heater.

NOTE: Conduct the hidden platform trial from days 2−6. If the mouse cannot find the platform in 60 s, lead the mouse to climb up to the platform and allow it to stay there for 10–30 s at the end of each trial. Perform four trials/day for each mouse for 5 consecutive days, with the platform and the visual cues at constant positions.

5.4. Perform the probe trial.

NOTE: Locate each mouse in the pool at a novel start position to observe the spatial exploration ability of the mouse.

5.4.1. Remove the platform.

5.4.2. Locate each mouse facing the tank wall in the pool once for 60 s. Ensure that the starting location is the NW quadrant, which is the quadrant furthest away from the SE quadrant.

5.4.3. Record the swimming distance, swimming speed, and the platform crossover number in the maze.

5.4.4. Dry each mouse with towels and provide warmth after the trial.

5.5. Perform the reversal trial.

NOTE: Perform the reversal trial from days 8−11.

5.5.1. Position the platform in the middle of the NW quadrant (instead of the SE quadrant).

5.5.2. Follow steps 5.3.2 −5.3.5 as detailed in the hidden platform trial section.

6. Statistical Analysis

6.1. Use statistics software (e.g., SPSS 20.0) to perform the statistical analysis.

REPRESENTATIVE RESULTS:

The time axis diagram of this protocol is shown in **Figure 1**.

[Place Figure 1 here]

The time axis shows that this experiment lasted for a total of 21 days. The treatment was applied to the mouse during the whole experiment and the MWM tests began after 15 days of treatment. The visible platform, hidden platform, probe, and reversal trials were conducted in order.

Previously published results from Ding et al.[5] are presented as typical results of MWM **Figure 2**.

[Place Figure 2 here]

Figure 2A shows the results of the visible platform trial. No statistical differences were observed in the escape latency or swimming speed among the groups on the first day of MWM. **Figure 2B** shows the results of the hidden platform and reversal trial from days 2−6 and days 8−11. The escape latency of the AD group remained at a high level on each day of the test. The escape latency of the other three groups decreased gradually. The escape latency from days 3−6 and days 8−11 was longer in the AD group than in the control group ($p < 0.01$). The escape latencies of mice in the MA and drug groups were shorter than that of mice in the AD group on days 2−6 and days 8−11, respectively ($p < 0.01$). **Figure 2C** shows the results of the probe trial. The platform crossover number of mice in the AD group was statistically lower than that in the control group ($p < 0.01$). The platform crossover number in the MA group was higher than that in the AD group ($p < 0.05$). The proportion of time spent in the SW quadrant by mice in the AD group was significantly lower than that in the control group ($p < 0.01$). The proportion of time spent in the SW quadrant in the MA group was higher than that in the AD group ($p < 0.01$).

FIGURE LEGENDS:

Figure 1: Time axis diagram of the study protocol.

Figure 2: Typical results of the Morris water maze test (n = 10). (**A**) Changes in the escape latency and swimming speed of rats among the different groups in the visible platform trial. (**B**) Changes in the escape latency of rats among the different groups in the hidden platform and reversal trials. The p-values are *$p < 0.05$ and **$p < 0.01$ compared with the control group. The symbol ## indicates $p < 0.01$ compared with the AD group. (**C**) Changes in the platform crossover number and the percentage of the time spent by the rats in the northwest quadrant among the different experimental groups in the probe trial. The results of the visible platform, hidden platform, and reversal trial in each group are shown (n = 10, mean ± SD). This figure has been modified from Ding et al[5].

DISCUSSION:

Although many water mazes, including the Biel water maze and the Cincinnati water maze, have been around for at least a century, only the MWM has been widely used to effectively and objectively evaluate spatial learning and memory ability because it has many advantages[9]. Despite extensive use of the MWM, the procedure has not always been used optimally. MWM experiments generally take a long time and are influenced by many variable factors. There are some effective and reliable aspects that help detect changes in spatial learning and memory ability that should be taken into consideration.

Four different MWM trials were performed. The visible platform trial was used on day 1 of MWM. If the animals could swim directly to the platform, it indicated that the swimming ability and vision of the animals were normal[10]. Otnass suggested that the visible platform trial should be conducted first[11]. The results of the visible platform trial in this study meant that the four groups started at the same learning level. From there, the successive experiments could be started. The hidden platform trial was used to assess the ability of the mice to acquire learning and memory ability. The probe trial was conducted on day 7, 24 h after the end of the hidden platform trial, to assess working memory. Finally, the reversal trial was used to assess working memory[2]. The changes in the four different trials of MWM together indicated that the AD model mice had low learning and memory ability and that MA had a positive effect on AD[5].

There are no specific standards for the dimensions of the pool and platform[1]. A 214-cm diameter pool is used in most MWM studies. Vorhees and Williams demonstrated that with identical protocols, rats learn faster in a 122-cm pool than in a 210-cm pool; the steep slope of the learning curve indicates that the 122-cm diameter pool is extremely easy for rats to navigate[12]. In the current protocol, considering the old age and weak stature of the AD mice, a 90-cm diameter pool and 9.5-cm diameter platform were used. The results of preliminary experiments indicated that mice had more difficulty finding the platform in a bigger diameter pool. Therefore, tests in larger pools do not represent the real difference among the groups. The experimental animals had a harder time finding the platform in a bigger pool with a smaller platform[4]. Therefore, the size of the pool and the platform must be optimized in preliminary experiments according to the experimental requirements and condition of the experimental animals.

Water at temperature ranging from 20–24 °C is recommended for performing the MWM test[4]. Aged experimental animals performed poorly in cold water[13], indicating a clear age-dependent loss of thermoregulation[14]. In this study, a thermostat was placed at the bottom of the pool to maintain the temperature of the water at 20–24 °C. The study results showed no significant difference in the swimming speeds among the four groups[5].

MWM is a powerful technique to assess cognitive function and is widely used in studies presently. However, there is no defined, standard, consistent equipment to perform the MWM test, including the sizes of the pool and platform[15,16]. Different laboratories have different specifications for MWM. Therefore, researchers choose the appropriate experimental device according to their individual experimental requirements, which may cause confusion among researchers. Preliminary experiments are also necessary. More studies need to be conducted on basic experiments like MWM. Presently, the flexibility of MWM as an experimental tool only

lies in the ability to choose the basic protocols according to the study purposed. Therefore, this test can be applied to assess cognitive function in greater depth.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no potential conflict of interest.

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Figure1

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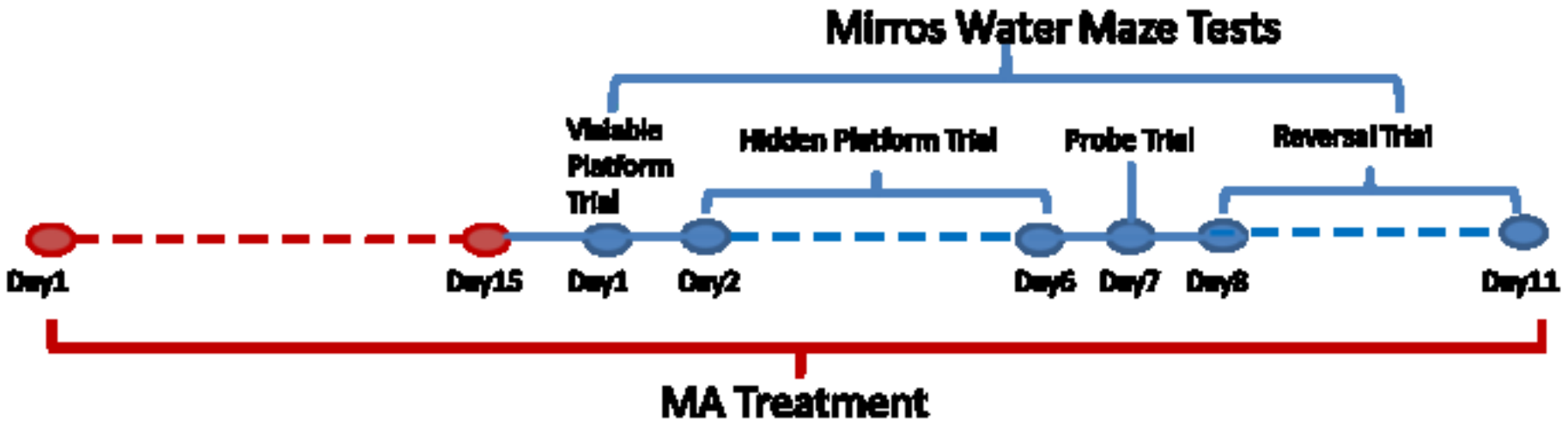

Figure2

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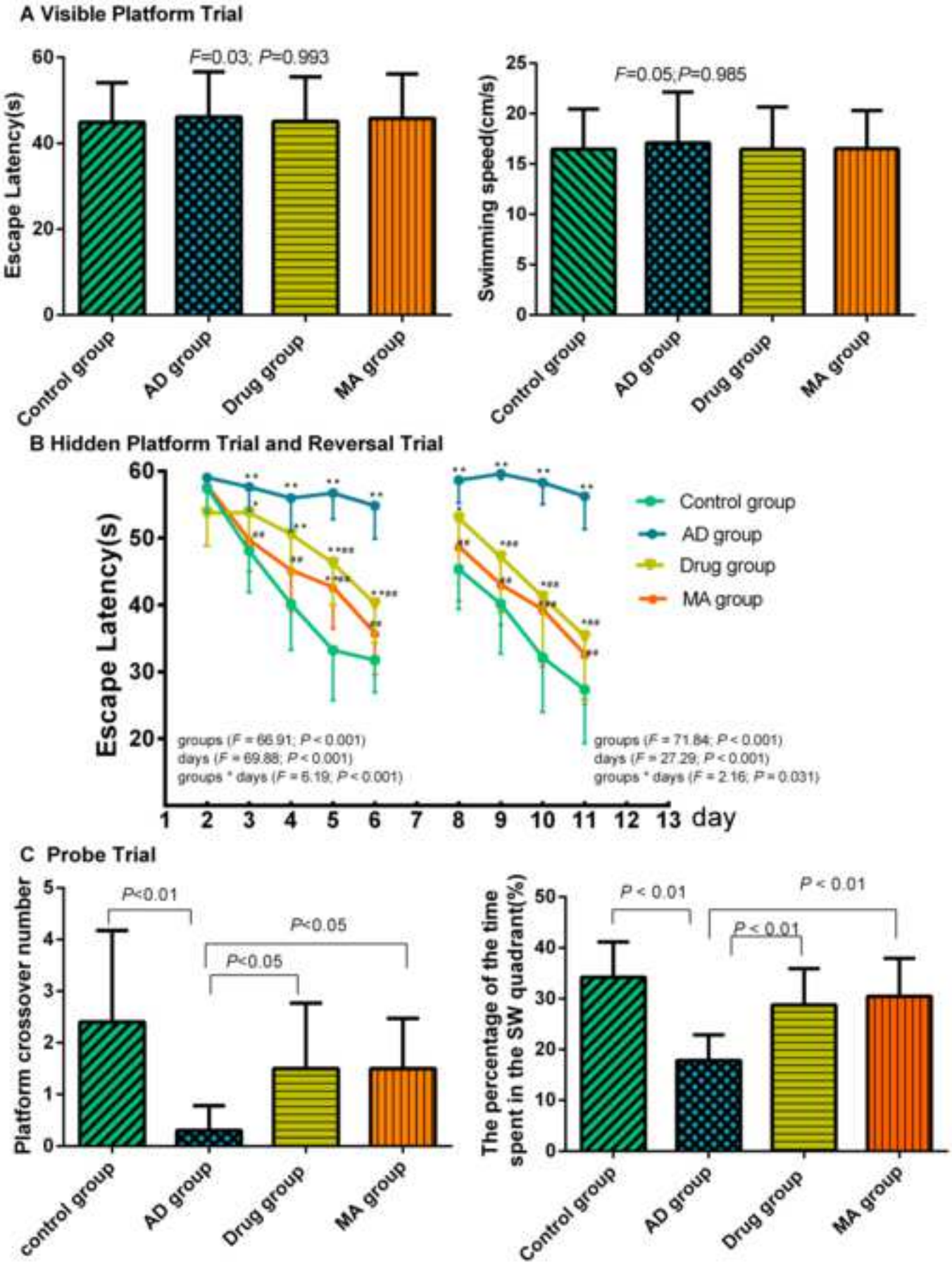

Name of Material/Equipment	Company	Catalog Number	Comments/Description
acupuncture needles	Beijing Zhongyan Taihe Medical Instrument Limited Company	511526	
desktop computer	Chengdu Techman Software Limited Liability Company	Lenovo T4700D	
Donepezil Hydrochloride Tablet	Eisai China	H20050978	Aricept
mice	Zhi Shan (Beijing) Academy of Medical Science	SCXK2014-0003	
Mirros water maze device	Chengdu Techman Software Limited Liability Company	WMT-100S	
mouse bags	home-made		
Signal acquisition and processing system	Chengdu Techman Software Limited Liability Company	BL-420N	

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Answer: I appreciate the opportunity you have given me to correct the errors.
The manuscript was edited by Editage, a professional English language editing service.

2. We cannot have punctuation marks in the title of the manuscript. Please reword the title to make it concise and in alignment with the protocol described in the text.
Answer: Thank you for your suggestion. Accordingly, I have changed the title.

3. Please provide an email address for each author.
Answer: The email address of each author has been provided as required.

4. Please provide at least 6 keywords or phrases.
Answer: I am very grateful to you for your suggestion. I have included 6 key words in the revised manuscript; “Morris water maze, Protocol, Behavior, Alzheimer’ Disease, Mouse, Manual acupuncture”.

5. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”.
Answer: Thank you! I have changed the Short Abstract to the following:

“Herein, we present a protocol to introduce how to conduct the Morris water maze tests to evaluate the ability of learning and memory of Alzheimer’s disease (AD) model mice and to assess the effect of manual acupuncture for treating AD model mice.”

6. The current Long Abstract is over the 150-300-word limit. Please rephrase the Long Abstract to more clearly state the goal of the protocol.
Answer: Thank you for your suggestion. The present Long Abstract (264 words) has been rephrased and revised within the 150-300-word limit.

7. Please define all abbreviations during the first-time use.
Answer: All abbreviations used have been defined in the manuscript.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Eisai China, Inc., H2005097, Beijing Zhongyan 97 Taihe Medicine Company, Ltd., Baihui (GV20) and Yintang (GV29), TOTA-450d, Japan, China Daheng Group, China, SPSS, Inc., Chicago, 173 IL, United States, EthoVision, etc.
Answer: Thank you very much. The use of commercial language has been revised.

9. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 34-36, 47-49, 77-82, 85-86, 103-107, 108-110, 112-113, 127-129, 154-155, 161-163, 172-182, 210-213, 218-221, 231-233, 236-238.
Answer: Thank you for your careful perusal of our manuscript. I apologize for the overlap. All suggested overlaps have been revised individually.

10. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).
Answer: Thank you. I have revised the corresponding reference numbers.

11. Please revise the Introduction to include all of the following with citations wherever applicable:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Answer: Thank you. I have revised the manuscript according to your suggestion.

12. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.
Answer: Thank you. I have revised the corresponding reference numbers, and moreover, as suggested, I have used Calibri 12 font size.

13. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures.
Answer: Thank you. I have revised the manuscript according to your suggestion.

14. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
Answer： Thank you. I have revised the manuscript according to your suggestion.

15. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. All paragraph if describing the action should be converted to a numbered step and if discussing details should be moved to the discussion section.
Answer: Thank you for your suggestion. I have changed the protocol in the revised manuscript

as per your suggestion.

16. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
Answer: Thank you. I have changed this in the manuscript.

17. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
Answer: Thank you. I have changed this in the manuscript.

18. Please ensure you answer the “how” question, i.e., how is the step performed?
Answer: Thank you. As per your suggestion, I have explained the “how” question in the revised manuscript.

19. Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

20. Lines 75-82, 103-110, 112-116,135-138, 171-182 : Please make action steps.
Answer: Thank you. I have changed this section as per your suggestion.

21. 1: What is AD, MA and M group? Why different mice strain are used for controls.
Answer: AD is the abbreviation of Alzheimer’ Disease, and has been defined at first use in the Long Abstract. MA is the abbreviation for the manual acupuncture group mentioned in section 2.1 of the revised manuscript. M is the abbreviation of Medicine group mentioned in section 2.1 of the revised manuscript.
SAMP8 is senescence accelerated mouse/prone-8 mouse while SAMR1 mice are models for anti-aging. SAMR1 mice are usually used as experimental controls for SAMP8 mice.

22. 3.2: Please explain the Baihui and Yintang what does these stand for along with tahe abbreviations.
Answer: I apologize for not explaining these terms. Thank you for your critical review of the manuscript. Baihui and Yintang are the names of acupoints. They do not represent abbreviation.

23. 3.3: how do you ensure this?
Answer: The experimenters were acupuncturists and the method described in section 3.3 is routinely performed operation by them. Therefore, we can ensure this.

24. 4.1.1: how is this done?
Answer: Thank you. I have changed the description of the method section in the revised manuscript.

25. 4.1.6: how is the analysis performed?
Answer: Thank you very much. The analysis was performed by SPSS, and this has been mentioned in the Statistical Analysis section of the revised manuscript.

26. After how long of the MA do you perform the test? Did you provide MA just once? Do you keep providing MA in between? Please bring out the link between the treatments and test.
Answer: Thank you! MA was applied during the experimental procedure, including the MA treatment and Morris water maze tests, and this information has been provided in the revised manuscript.

27. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Answer: I am very grateful for your suggestion. The filmable content is the step 5 of the protocol and we have ensured that it is less than 2.75-page length.

28. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.
Answer: Thank you. The embedded figure has been moved from the manuscript to individual files and uploaded separately.

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Answer: Thank you.

30. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.
Answer: The figure legends have been provided after the results section as suggested.

31. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

32. Please do not abbreviate the journal titles in the references section.
Answer: Thank you. I have provided the full names of journals in the reference section.

33. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns.

Answer: Thank you. I have revised this section.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The research was interesting and the paper has been written in a clear way.

Major Concerns:
But the similar researches have been published earlier, hence the current research seems a repeating one, lacking of a certain creation.
For example, a paper "Effects of electro-acupuncture on the behavior and the expression of ADAM10 and ADAM17 in cerebral cortex of SAMP8 mice" published on "China Journal of Traditional Chinese Medicine and Pharmacy", written by WANG Xin, PIAO Zan-xun, ZHOU Yuan, LI Zhi-gang, XU An-ping in 2016,31(10):4006-4010.
Another paper "Impacts of Electroacupuncture on the Behavior in SAMP8 Mice and the Expression of β Amyloid Precursor Protein Cleaving Enzyme in Cerebral Cortex" published on "World Journal of Integrated Traditional and Western Medicine" by XU An-ping, LI Zhi-gang, TANG Yin-shan, MO Yu-ping, YAO Hai-jiang, SONG Hong-tao, Saiyin Chaoketu in 2014, 09(02):192-195.
The above published papers use the same mouse, the same grouping, the same acupoints, the same number in each group, and the same treatment course, 15 days. The above papers also observed the behavior changes of the mouse, including the Morris water maze.

And the other papers published recently, also similar to the current research. Maybe used the stimulation of manual acupuncture instead of electric stimulation.
Ding N, Jiang J, Xu A, et al. Manual Acupuncture Regulates Behavior and Cerebral Blood Flow in the SAMP8 Mouse Model of Alzheimer's Disease. Front Neurosci (Switzerland), 2019, 13 p37
Jiang J, Liu G, Shi S, et al.
Effects of manual acupuncture combined with donepezil in a mouse model of Alzheimer's disease [epub ahead of print] Acupunct Med (England), Mar 07 2019, pacupmed2016011310

The current paper recorded the behavior changes in details, more details than those of the above published papers. But usually new ideas is worth to be read, while repeating observation has less attraction.
Answer: I am very grateful to you for your kind suggestion. Visual experimental protocols are extremely essential for researchers. Therefore, we used the latest published study as an example to introduce the protocol of the MWM in this paper.

Reviewer #2:
The following should be added into paper：
What is the age of mice? Male or female?
Answer: Thank you for your suggestion. The male mice used in the experiments were 8 months

old.

Acupuncture direction?
Answer: Thank you for your suggestion. I have included the following revision: “apply MA on Baihui (GV20) and Yintang (GV29) using flat thorn method toward nose for 20 min.”

What is the direction of the mouse's entry into the water
Answer: The mouse entered the water facing the wall of the tank.

The amount of milk powder?
Render the water opaque with about 150 g of milk powder.
The basis and limitation of the selection of evaluation parameters can be added to the discussion.
Answer: Thank you. We have taken this suggestion into consideration.

Reviewer #3:
Manuscript Summary:
The paper reports the effects of acupuncture on the animal model of cognitive impairment, as assessed by Morris water maze as a spatial memory test. Clear and synthetic, the paper describes in details the experiment and its results.

Major Concerns:
None

Minor Concerns:
It could be useful to add, in the discussion, some considerations about acupuncture mechanism of action, and references regarding eventual further studies implying this technique for that kind of neurological disorder.
Answer: We appreciate your suggestion. We have considered your suggestion carefully and revised our discussion as suggested. Thank you once again.

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Title of Article: Morris water maze: Assessing the effect of manual acupuncture on the learning and memory of SAMP8 Mouse

Author(s): Huiling Tian,Ning Ding, Jing Jiang, Mengwei Guo,Shun Wang,Yujie Li, Zidong Wang,Hao Liu,Jiayi Yang,Jingyu Ren,Zhigang Li.

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