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Corresponding Author:	Dong Kun Lee, Ph.D. Institute of Health Sciences Jinju, Gyeongsangnam-do KOREA, REPUBLIC OF
Corresponding Author's Institution:	Institute of Health Sciences
Corresponding Author E-Mail:	dklee@gnu.kr
Order of Authors:	Hyeonwi Son
	Ju Hwan Yang
	Hyun Joon Kim
	Dong Kun Lee, Ph.D.
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TITLE:

A Chronic Immobilization Stress Protocol for Inducing Depression-Like Behavior in Mice

AUTHORS AND AFFILIATIONS:

Hyeonwi Son[1], Ju Hwan Yang[2], Hyun Joon Kim[1]*, and Dong Kun Lee[3]*

[1]Department of Anatomy and Convergence Medical Sciences, Institute of Health Sciences, Bio Anti-aging Medical Research Center, Gyeongsang National University Medical School, Jinju, Republic of Korea

[2]Department of Biological Sciences, Pusan National University, Busan, Republic of Korea

[3]Department of Physiology and Convergence Medical Sciences, Institute of Health Sciences, Gyeongsang National University Medical School, Jinju, Republic of Korea

*These authors contributed equally.

Corresponding Authors:

Hyun Joon Kim (kimhj@gnu.ac.kr)

Dong Kun Lee (dklee@gnu.ac.kr)

Email Addresses of Co-authors:

Hyeonwi Son (hw.son@gnu.ac.kr)

Ju Hwan Yang (ssozooaa@gmail.com)

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SUMMARY:

This article provides a simplified and standardized protocol for induction of depressive-like behavior in chronically immobilized mice by using a restrainer. In addition, behavior and physiological techniques to verify induction of depression are explained.

ABSTRACT:

Depression is not yet fully understood, but various causative factors have been reported. Recently, the prevalence of depression has increased. However, therapeutic treatments for depression or research on depression is scarce. Thus, in the present paper, we propose a mouse model of depression induced by movement restriction. Chronic mild stress (CMS) is a well-known technique to induce depressive-like behavior. However, it necessitates a complex procedure consisting of a combination of various mild stresses. In contrast, chronic immobilization stress (CIS) is a readily accessible chronic stress model, modified from a restraint model that induces depressive behavior by restricting movement using a restrainer for a certain period. To evaluate the depressive-like behaviors, the sucrose preference test (SPT), the tail suspension test (TST), and the ELISA assay to measure stress marker corticosterone levels are combined in the present experiment. The described protocols illustrate the induction of CIS and evaluation of the changes

in behavior and physiological factors for the validation of depression.

INTRODUCTION:

Major depressive disorder (MDD) is the leading cause of mental disability worldwide, with an incidence that is increasing faster than anticipated. In 2001, the World Health Organization predicted that MDD would be the second most common disease in the world by 2020. However, it was already the second most common in 2013[1]. In addition, current antidepressants have many limitations, including delayed effectivity, drug resistance, relapse, and various side effects[2,3]. Researchers must therefore develop more effective antidepressants. However, the ambiguous pathophysiology of MDD presents an obstacle to the development of novel antidepressants.

Long-term stress is the main risk factor for MDD. It can induce dysfunction in the hypothalamic-pituitary-adrenal (HPA) axis, which is also related to MDD etiology[4,5]. As described previously, the HPA axis plays a critical role in stress-induced psychiatric pathophysiology including depression and anxiety disorders by increasing corticosterone levels[6–9]. Many animal models have been based on sustained activation of the HPA axis, which is observed in patients with MDD[4]. Moreover, high glucocorticoids induced by chronic stress and subcutaneously injected glucocorticoids cause depressive behaviors along with neural cell death, atrophy of neuronal processes, and reduced adult neurogenesis in the brain of rodents[10,11]. Another important brain area associated with depression is the medial prefrontal cortex (mPFC). The mPFC plays a crucial role in controlling brain subregions, such as the hypothalamus and amygdala, that control emotional behavior and stress responses[8,9]. For instance, lesions in the dorsal mPFC induced HPA axis dysfunction and enhanced corticosterone secretion due to restraint stress[12,13]. A recent study also showed that repeated restraint stress increased corticosterone levels, which could be decreased by glutamine supplementation via glutamate-glutamine cycle between neurons and astrocyte in the mPFC[9].

The first chronic stress paradigm used to study the etiology of MDD was suggested by Katz[14]. Willner et al. then proposed a chronic mild stress (CMS) model based on the findings of Katz. They confirmed that the model had predictive validity by observing that antidepressants restored CMS-induced anhedonic-like behavior[15,16]. Typically, the CMS model consists of a combination of various mild stresses, such as mild noise, cage tilting, wet bedding, altered light-dark cycles, cage shaking, forced swimming, and social defeat. The CMS model is widely utilized by researchers; however, this model is of poor replicability, and time- and energy-inefficient. Therefore, there is a growing demand for a standardized and simplified protocol for induction of depressive-like behavior and physiological analysis to evaluate depression. Compared to the CMS model, the chronic immobilization stress (CIS; also known as chronic restraint stress) model is simpler and more efficient; therefore, the CIS model can be widely used in chronic stress studies[17–24]. In addition, CIS can be used in both male and female mice to develop depressive behaviors[25,26]. During CIS, animals are placed in a body-fit sized cylinder for 1–8 hours per day for 2 or 4 weeks[9,27,28]. Of these, restraint stress condition for 2 hours per day for 2 weeks is sufficient to cause depressive behaviors with minimal pain in mice[9,28]. Under restraint conditions, blood corticosterone levels were rapidly increased[9,28,29]. Several studies have shown that the CIS model has predictive validity, confirming that CIS-induced depressive-like symptoms are restored by

antidepressants[19,20,30,31]. Herein, we report the detailed procedures of CIS, as well as some behavioral and physiological outcomes after CIS in mice.

PROTOCOL:

All experimental protocols and animal care were conducted according to the guidelines of the University Animal Care Committee for Animal Research of Gyeongsang National University (GLA-100917-M0093).

1. Materials

1.1. Mice

1.1.1. Use males of C57BL/6 strain background weighing 22–24 g at postnatal week 7. Habituate in the breeding room for 1 week before the experiments.

NOTE: All mice were purchased from a laboratory animal company.

1.1.2. House mice individually in a temperature-controlled vivarium (22–24 °C) under a 12-hour light/dark cycle (lights on at 6:00 A.M.), with normal laboratory chow and water available ad libitum.

1.2. Restrainer

1.2.1. Use a cylindrical, transparent, acrylic tank (height = 8.5 cm, diameter = 2.5 cm) fixed on a square pedestal to restrain and to produce depressive behavior (**Figure 1A**). The diameter of this cylinder was made to fit the body so that the mouse could not turn and move forwards or backwards. The restrainer can be purchased commercially or made in the lab.

1.3. Tail suspension apparatus

1.3.1. Use a reasonable size tail suspension box made of translucent acrylic (height = 30 cm, width = 20 cm, length = 20, **Figure 1B**). To prevent interactions between the animals, use rectangular partitions within the box so that the floor and three of the four walls are blocked by acrylic plates. Leave the remaining two sides of the box open to allow video recording and to fix the horizontal bar. The box can be purchased commercially or made in the lab.

1.4. Video recording device and video tracking software

1.4.1. Use a black and white-display closed-circuit television camera (see **Table of Materials**) connected to a computer and a tripod (or other support products) to allow recording of the behavioral experiment. Video recording is essential to allow behavioral scoring in this experiment, because at least two mice are tested at the same time.

1.4.2. Ensure that the camera resolution is high enough to allow the video data to be analyzed using the video tracking software (see **Table of Materials**) installed in the connected computer.

2. Induction of depression by CIS restraint

NOTE: Handle the mouse gently, but firmly with confidence. Both rough and tentative handling is another stress factor in the experiment and it is an important reason for the mouse struggling, biting, and scratching.

2.1. Set the room light to **light (200 Lux)** conditions using a digital lux meter.

2.2. House the mouse in a separate cage at least a week before testing and place the mouse in the testing room for at least 30 min before the experiment.

NOTE: Handle the mice at least once a day for at least 3 consecutive days before the experiment so that the mice become familiar with the experimenter. An adaptational period before the experiment is necessary to ensure that the mice acclimatize to the circumstance, such as the testing room.

2.3. Gently hold the mouse tail to avoid tensing the mouse, and then carefully place it on a rough surface (top of wire bar of the cage or cage lid).

2.4. Cover the restrainer with a small white towel, and then gently place the mouse at the opening of the restrainer so that the mouse enters the restrainer voluntarily.

NOTE: In this case, the mouse is positioned in the opposite direction to that which it enters the restrainer with. To lead the mouse to enter the restrainer voluntarily, the restrainer is covered with a small towel to make the inside darker.

2.5. Place the closure to restrain the mouse as tight as possible, being careful to avoid damage to the body, such as tail, feet, and testicles.

2.6. Restrain the mouse for 2 hours/day (9:00 A.M. to 11:00 A.M.) for 15 consecutive days.

2.7. Measure body weight and food intake every 48 hours during exposure to the restrainer (i.e., food intake quantity during the 48 hours before the initiation of the movement restraint).

NOTE: When measuring body weight and food intake, place control mice in their home cages in the testing room during CIS. Ensure that other environmental factors are the same as for the CIS mice.

2.8. Confirm the induction of depression by performing behavioral tests such as the sucrose preference test (SPT) and the tail suspension test (TST) (refer to steps 4 and 5).

2.9. Confirm the induction of depression by measuring the stress marker corticosterone using ELISA assay (refer to section 6).

3. The sucrose preference test

3.1. Before testing, habituate the mice to the presence of two drinking bottles (one containing 0.1 M sucrose and the other containing plain water) for 48 hours. Switch the positions of the two bottles after 24 hours to reduce any confounding produced by a side bias.

3.2. On the 3rd day, deprive the mice of water for 24 hours.

3.3. On the day of the SPT experiment, expose the mice to two drinking bottles for 6 hours. After 3 hours, switch the position of the water bottles.

3.4. Record the volume (mL) of sucrose solution and water consumed and then calculate the animals' affinity to sucrose.

3.5. Generally, calculate sucrose preference as a percentage of the volume of sucrose consumption over the total fluid consumption during the test.

4. The tail suspension test

4.1. Bring the CIS-induced mice into the testing room at least 30 minutes before beginning the TST.

4.2. Set the room light to **dim (50 Lux)** conditions.

4.3. To obtain the highest resolution video file, place the camera as close to the mouse as possible (around 40 cm from the mouse).

4.4. Suspend the mouse firmly from the horizontal bar (30 cm from the bottom line) using cellophane adhesive tape (the distance from the tip of the tail is 1 cm). Complete the process of applying tape to the mouse as soon as possible to minimize other sources of stress.

4.5. Once the mouse is firmly suspended from the horizontal bar, place it in the middle of the suspension box and fix the bar with tape to prevent struggling.

4.6. Once the mouse is positioned in the middle of suspension box, start recording and observe the behavioral alterations continuously for 6 minutes. If the mouse attempts to climb its tail, use a stick or climb stopper to prevent it from doing so.

4.7. At the end of the experiment, move the mouse to its home cage and carefully remove the tape from its tail.

4.8. Analyze the accumulated time of immobile periods using the video tracking software.

NOTE: The duration of immobility is the most important CIS parameter. This can be calculated as the accumulated time of immobile periods, defined in terms of a motion threshold contained within the level-filtering device of the software.

5. **Measuring corticosterone levels in blood by ELISA**

NOTE: A day after the behavioral test, the mice are sacrificed for blood collecting.

5.1. Anesthetize the mouse with 5% isoflurane in an induction chamber until anesthesia. Ensure the mouse has sufficient time in the induction chamber (at least 2 minutes) to prevent waking up during surgery.

5.2. Collect blood from the heart using a 1 mL syringe, and store the blood in vacutainers containing K_3EDTA on the ice (at 9 A.M.)

5.3. Separate plasma by centrifugation at 1,000 × *g* for 15 min at 4 °C.

5.4. Quantify plasma corticosterone levels using the corticosterone ELISA kit (see **Table of Materials**) according to the manufacturer's protocol.

REPRESENTATIVE RESULTS:

In the representative experiment, all data were acquired from 6 - 8 mice per group. Representative materials and the method to insert the mouse voluntarily into the restrainer are shown in **Figure 1**.

To perform the behavioral test and blood sampling after CIS induction, mice were subjected to the experimental procedure as summarized in **Figure 2A**. As shown in **Figure 2** and **Figure 3**, CIS induces depressive-like behaviors well and releases the stress marker corticosterone. In addition, these indexes were recovered by glutamine supplementation (mice were fed glutamine-supplemented diets during the entire experimental period, 150 mg/kg) as shown in **Figure 3**.

FIGURE AND TABLE LEGENDS:

Figure 1: Restrainer setup. (A) Restrainer, **(B)** tail suspension box, and **(C)** water bottle and ball nozzle. **(D)** The process of inserting the mouse into the restrainer to induce CIS. From the left panel, mouse voluntarily enters the restrainer after the restrainer is covered with a small towel. The right panel shows that the mouse has completely entered the restrainer. This figure was modified from Son et al.[9] Copyright permission has been obtained from the journal for all reused figures.

Figure 2. Induction of the chronic immobilization stress and evaluation of depressive-like behaviors in mice. (**A**) Experimental procedure. Body weight (**B**) and food intake (**C**) in the control group (blue line, n = 8) and in the CIS group (red line, n = 8). (**D and E**) Sucrose preference and

immobility time (n = 8 in both tests). **(F)** Blood corticosterone levels (n = 7/group). Data are shown as mean ± SEM. *p < 0.05 as determined by (**B** and **C**) two-way ANOVA with Bonferroni *post-hoc* test or (**D–F**) unpaired Student's t-test. CIS = chronic immobilization stress, SPT = sucrose preference test, TST = tail suspension test, DC = decapitation. This figure was modified from Son et al.[9] Copyright permission has been obtained from the journal for all reused figures.

Figure 3. A glutamine-supplemented diet ameliorates depressive-like behaviors. Body weight (**A**) and food intake (**B**) in the control group (blue line, n = 7), CIS group (red line, n = 7) and CIS + glutamine supplemented group (green line, n = 7). Sucrose preference **(C),** immobility time **(D)** and blood corticosterone levels **(E)** (n = 6-7/group). Data are shown as mean ± SEM. *p < 0.05 as determined by (**A** and **B**) two-way ANOVA with Bonferroni *post-hoc* test or (**C-E**) unpaired Student's t-test. Gln = glutamine. This figure was modified from Son et al.[9] Copyright permission has been obtained from the journal for all reused figures.

DISCUSSION:

The complexity of the brain and heterogeneity of MDD make it challenging to create animal models that completely reproduce the condition. Many researchers have overcome this difficulty using an endophenotype-based approach[32], in which anhedonia (lack of interest in rewarding stimuli) and despair are considered evolutionarily conserved and quantifiable behaviors in animal models, which are also seen in patients with depression[33]. In the present paper, we have presented a method in which CIS was sufficient to induce anhedonia and despair, demonstrating translational relevance between CIS and MDD. Moreover, many studies have used CIS to identify the mechanism eliciting depressive-like behaviors and to assess antidepressants capable of restoring normal behavior[9,19,20,30,31,34]. Thus, the CIS may be appropriate for studying the etiology of MDD and may therefore be useful in the development of new antidepressants.

Several factors affect the development of depressive-like behavior during CIS. The first is animal strain because the extent of stress response to CIS may vary depending on the animal strain. Indeed, several strain-related differences in response to depressive behavioral tests and antidepressants are known[35,36]. In this regard, particular attention should be paid to the tail-climbing behavior of the commonly used C57BL strain[37–39]. Second, environmental stress factors, such as light, noise, and housing, should be minimized. Although social isolation stress may influence the findings[40,41], we conducted CIS on single-housed mice because isolation has more advantages than disadvantages. For example, it can minimize social defeat stress because CIS often causes group-housed mice to attack each other. Indeed, the control mice also attack their housemates, affecting the baseline behavior in the TST and SPT. Another factor to consider before starting the experiment is sex. In this article, we performed all experiments with male mice, as emotional and cognitive behaviors are affected by the menstrual cycle in female mice[42–44]. Moreover, female rodents are relatively more susceptible to stress-related disorders, such as depression. Therefore, if the experimenter wants to use female mice, the time point of depressive-like behavior induction should be confirmed and the CIS protocol should be modified. In addition, all mice should be allowed a period of habituation to the new circumstance, and the experimenter should avoid adding new animals to the testing room during the experiment, because the mice may sense new olfactory and ultrasonic cues during the experiment. When the

experimenter is moving the mice to another floor or a long distance, it is necessary to cover the breeding cage with a piece of black cloth. Lastly, age is an important factor in determining the extent of response and recovery to stress[45]. We focused on the etiology of MDD in adolescence—8-week-old mice were used throughout the experiment. Experimenters should consider whether the abovementioned factors may influence the results when designing the CIS.

To validate CIS induction, tests that indicate depression, such as body weight and food intake measurement, TST, and SPT, should be performed and a physiological stress indicator, such as changes in corticosterone, should be investigated[9,46,47]. However, the TST method applied in this experiment is not recommended in rats because rats are too heavy to be supported by their tails. In such cases, the TST should be replaced with forced swimming or open field tests[39,48]. In this experiment, the primary consideration was the size of the suspension box. By using adhesive cellophane tape, the tail of the mouse was suspended on a horizontal bar located in the middle of the box on the ceiling. Therefore, the box should be large enough to prevent the mouse from contacting the wall during the experiment. The SPT, an indicator of anhedonia, suggests an emotional disorder such as depression. In the present experiment, the interest of the mice in a sweet drink was evaluated by using sucrose.

In order to induce depressive-like behavior, we modified the restraint technique of the CMS model to establish CIS, which is a simplified and highly reproducible technique to perform experiments in depression. However, in using CIS as a repetitive restraint model, there is a possibility that experimental animals could adapt to CIS and become insensitive to it. In addition, locomotion tests may not be appropriate as prolonged restraint could affect the movement of animals. Therefore, the establishment of a set point of restraint time in a day and consecutive days is important to minimize other factors except depression. In addition, it is necessary to perform the behavioral and the physiological test to verify the induction of depression after exposure to CIS.

In conclusion, despite the increasing interest of researchers in depression, it remains challenging to systematically define the pathological mechanism, which can be ascribed to the diverse and complex pathophysiology of depression. Hence, simplified animal models to induce depression, such as CIS, may provide important evidence to establish the mechanism of depression induction and suggest a good experimental platform to obtain therapeutic answers for such a complex mental problem.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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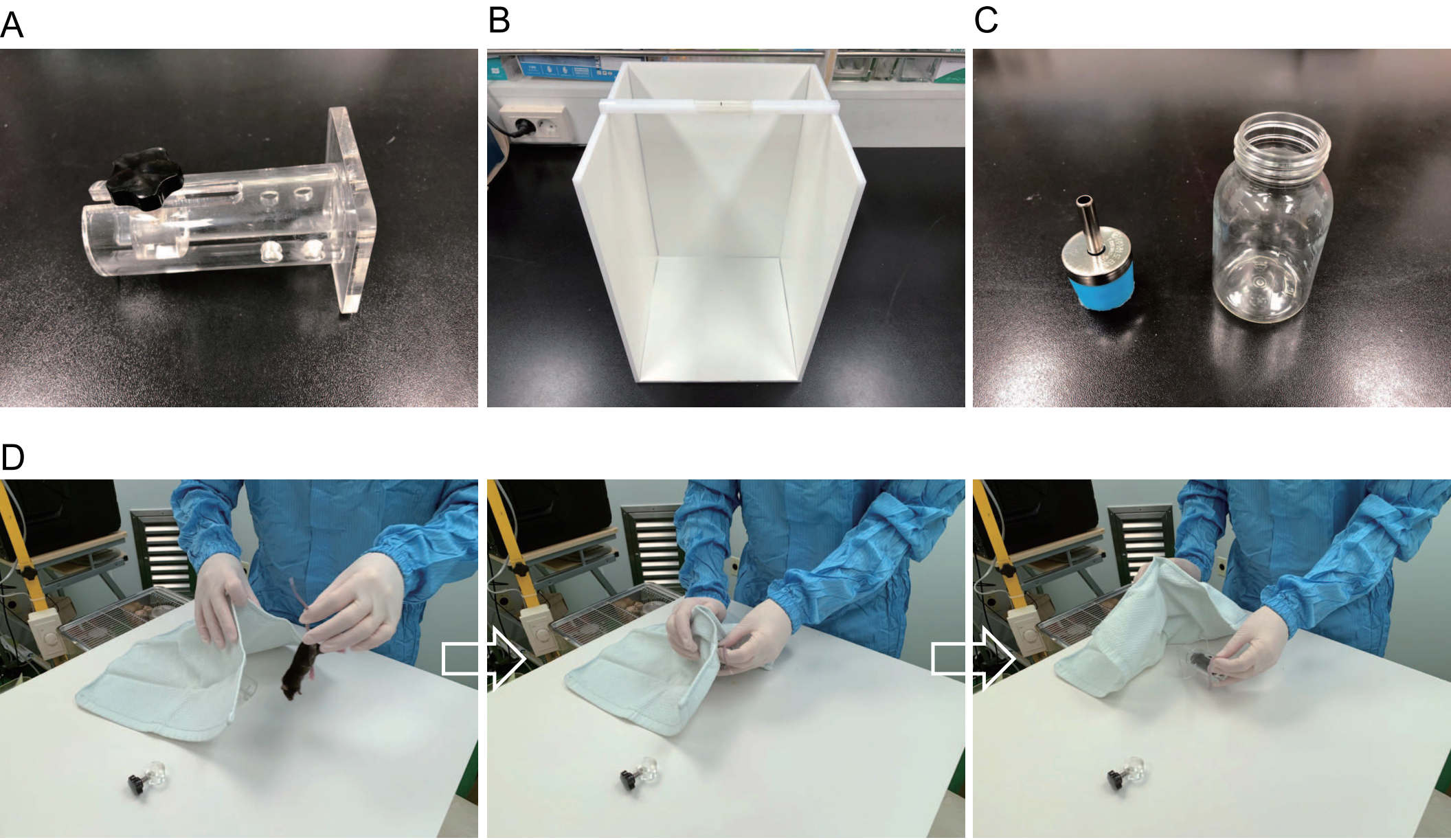


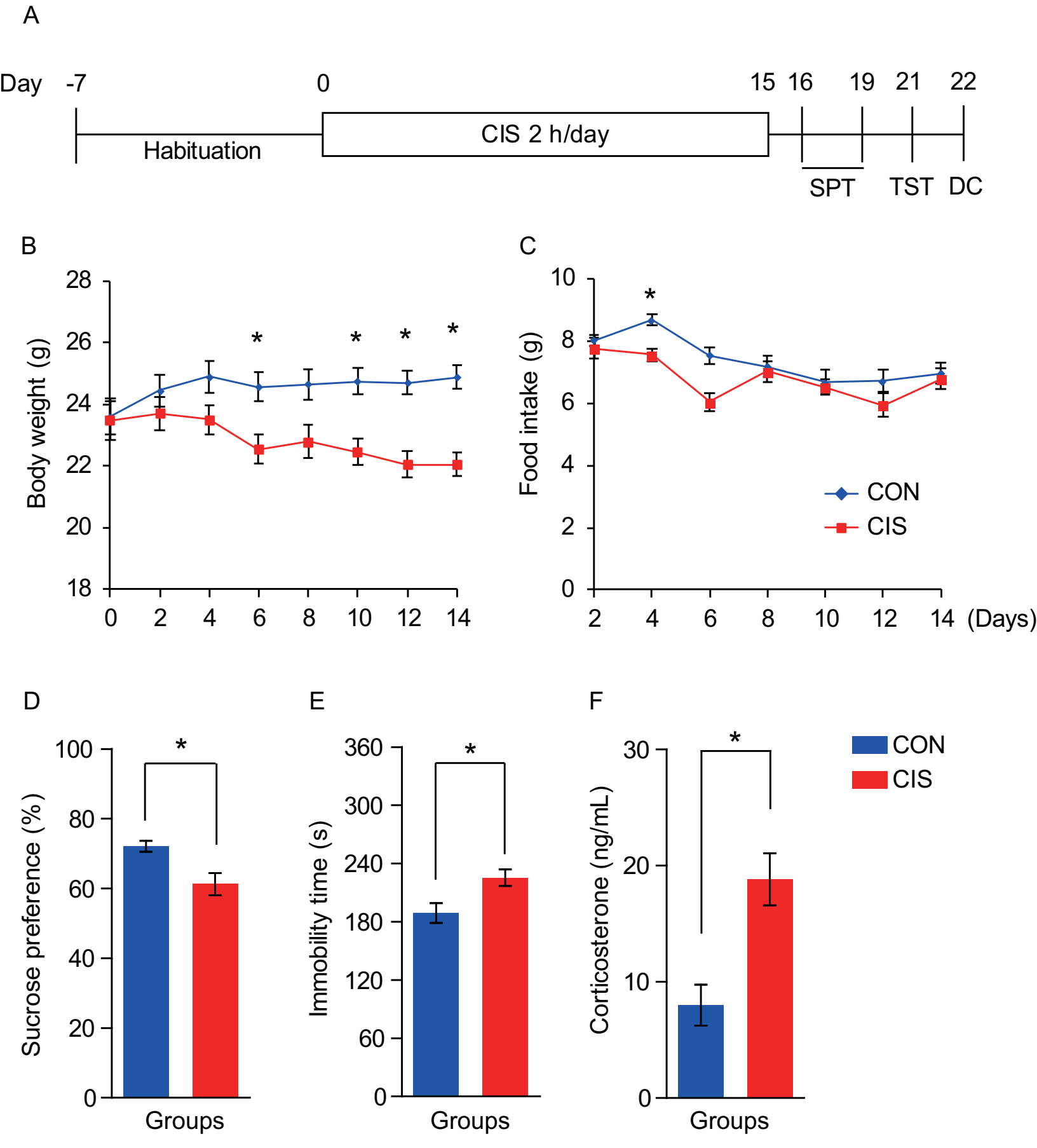
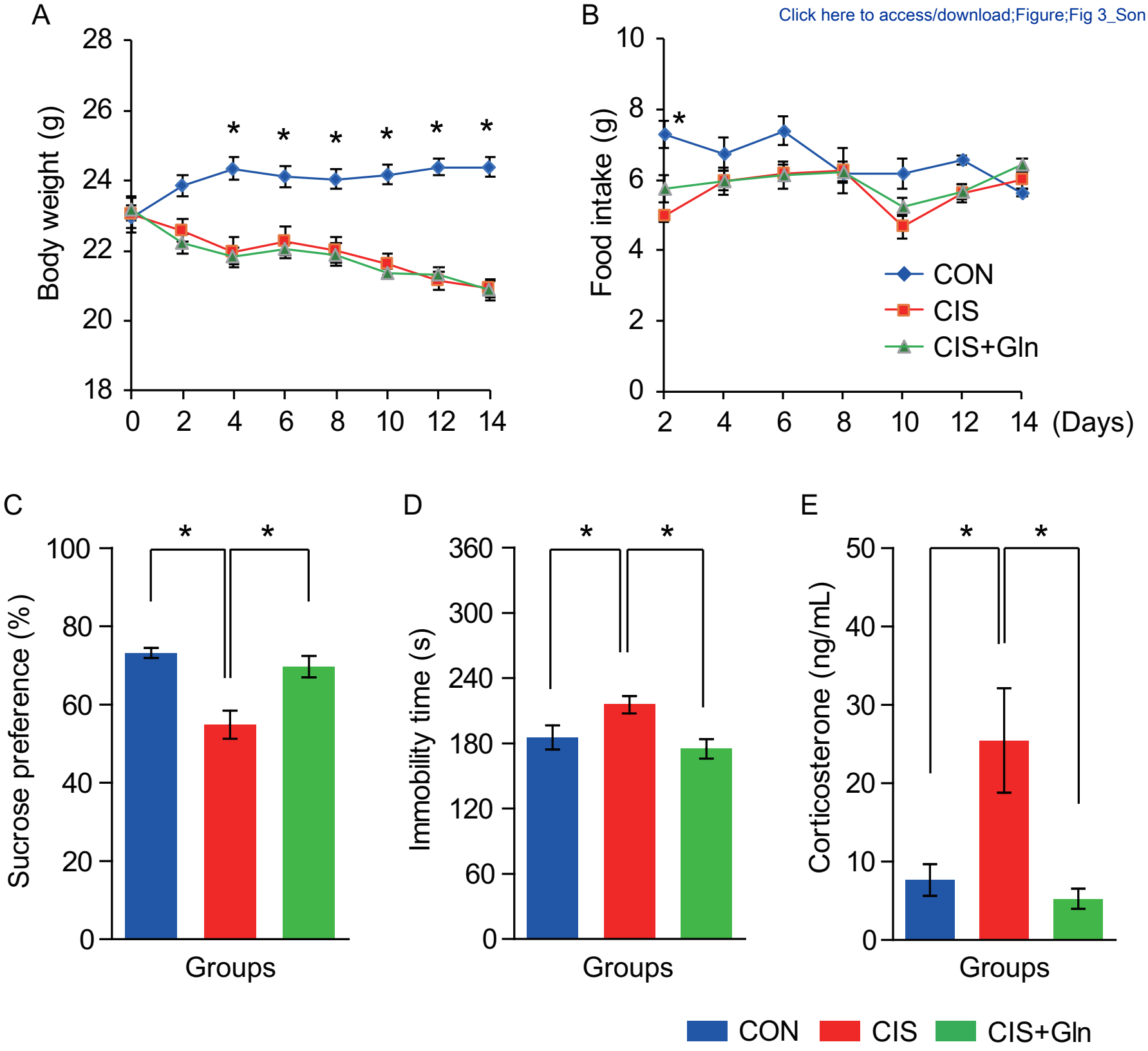


Figure 3



Name of Material/ Equipment	Company	Catalog Number
1 ml disposable syringes	Sungshim Medical	P000CFDO
Balance	A&D Company	FX-2000i
Ball nozzle	Jeung Do B&P	JD-C-88
CCTV camera	KOCOM	KCB-381
Corticosterone ELISA kits	Cayman Chemical	
Digital lux meter	TES	TES-1330A
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Mice	Koatech	
Restrainer	Dae-jong Instrument Industry	DJ-428
Saccharose (sucrose)	DAEJUNG	7501-4400
Small animal isoflurane anaesthetic system	Summit	
Acrylic bar		
Tail suspension box		
Timer	Electronics Tomorrow	TL-2530
Water bottle	Jeung Do B&P	JD-C-79

Comments/Description
Ifran solution
C57BL/6 strain
The apparatus was made in the lab for TST test
The apparatus was made in the lab

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Author(s):	Hyeonwi Son, Ju Hwan Yang, Hyun Joon Kim and Dong Kun Lee

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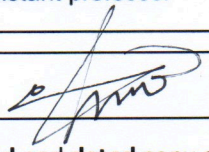
Department:	Department of Physiology of Gyeongsang National University Medical School
Institution:	Institute of Health Sciences
Title:	Assistant professor
Signature:	
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January 21, 2019

Dear Editor,

Reference; JoVE59546

Thank you for giving us the opportunity to revise our manuscript. Valuable revises of reviewers have made our article more compelling.

In this manuscript we have modified the overall part of the manuscript, including figures, introduction, discussion and protocol contents following the comments of the reviewers. Among major comment, we have accepted comments that refer to lack of novelty in the methodology and lack of detailed description. Thus, we have added figures of a novel method of CIS induction to minimize other stress using a restrainer in Fig. 1. And CIS-mediated changes in corticosterone levels, a stress indicator, and recovery effect of glutamine in CIS-induced depressive behavior were added in Fig. 2 and 3.

We really appreciate for the opportunity to revise our manuscript through following valuable comments through the revision work. All comments were critical and suggested valuable directions to correct the manuscript. Thank you again.

Sincerely,

Signature

Dong Kun Lee, Ph.D.
Assistant professor of Physiology and Health Sciences,
College of Medicine of Gyeongsang National University
dklee@gnu.ac.kr

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Changes to be made by the author(s) regarding the manuscript:
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Thank you for your concern. The manuscript was thoroughly corrected. Incorrect grammar and spelling have been corrected.

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3. Figure 1A: Please insert a space between “2” and “h/day” (i.e., 2 h/day).

Thank you for your comment. All mistakes in the manuscript and the figures were corrected following reviwer’s comment.

4. Affiliations: Please ensure that numbering follows the order of authors. First author gets 1, next author with different affiliation gets 2, etc., following from first to last.

The order of affiliations has been rearranged following the order of authors.

5. Please expand the Summary to briefly describe the applications of this protocol.

We really appreciate your comment. To expand the summary, brief introduction of the CIS is added, and verifying techniques to evaluate depression also added.

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Thank you for your comment. All commercial language were removed and all commercial products are referenced in the Table of materials in the manuscript following reviwer’s comment.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Thank you for your comment. All mistakes were corrected in the protocol text following reviwer's comment.

8. Please revise the protocol (lines 77-119) to contain only action items that direct the reader to do something (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." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Thank you for your comment. All mistakes were corrected in the protocol text following reviwer's comment.

9. Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.

Thank you for your comment. Introductory paragraphs of the protocol moved to the Introduction and Discussion, appropriately.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Thank you for your comment. The protocol has been simplified and every steps contain just 2-3 actions per step within 4 sentences.

11. 2.2.3: Please list an approximate distance used in the protocol.

Thank you for your comment. 2.2.3 moved to 5.3 and the approximate distance was listed in page 9, line 187.

12. Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

Thank you for your comment. Highlighted part of the protocol was corrected to include at least one action.

13. Please expand the Representative Results to describe how these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol.

Thank you for your comment. Representative results were expanded and modified to describe technique, suggestions to verify the induction of depression.

14. Discussion: Please discuss any limitations of the technique.

Thank you for your comment. The limitations of this technique is discussed from line 298 to line 301 in page 14.

15. Please ensure the Table of Materials has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

Thank you for your comment. All mistakes in the table were corrected following reviewer's comment.

16. References: If there are six or more authors, list the first author and then "et al.".

Thank you for your comment. References were corrected following reviewer's comment.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
In this manuscript the authors describe a method for chronic immobilization stress to induce despair in mice. This is an established model with multiple variations. There are a number of areas where additional detail could be included. The authors should imagine themselves having to do this method using on the details included in the manuscript. If they take such an approach they will see that the current details are insufficient for a methods paper. Beyond the lack of detail the other major issue with this paper is the lack of novelty or necessity for a video. Restraint tubes for mice is about as uncomplicated as one can get for a behavioral test and TST/SPT has been described elsewhere extensively.

Major Concerns:
* Missing from the introduction are any details on the physiological impact of restraint stress (acute or chronic). The authors should include a description of the impact on physiology (HPA axis responses, HR, etc) as there is likely both a physiological and psychological impact on the mice.

We appreciate the reviewer's comments. The physiological impact of restraint stress and the importance of brain region in depression research is newly described throughout the page 4 and 5, line 58-72.

* Since the reported method occurs in male mice only, the authors should address in the introduction the literature with CIS in males vs females and include in the discussion a description of sex as a limitation in the stated method.

We appreciate the reviewer's comments. Evidence CIS models in both male and female rodent is briefly describe in introduction at line 84 in page 4. And the considerations when choose mice gender to perform CIS is described in Discussion from line 271 to 276 in page 13.

* How do you put the mice in the restrainer tubes? What are the considerations of the choice of this method?

Thank you for your comment. The way to put the mice in the restrainer is newly described in the protocol step 3. And the advantages and considerations of the choice of this method are described from line 80 to 84 in page 4.

* In materials there are no details for the sucrose preference test. Also this is probably better described as a "two-choice preference test" or "choice preference test" since you are not actually using sucrose. Why use saccharin over sucrose?

Thank you for your critical comment. Actually, we use sucrose, but confused the terms saccharose (another synonym of sucrose) and saccharin, and described it as saccharin in the text. The incorrect words of the text have been corrected.

* Given that representative results show SPT before TST it is odd that the described method is TST and then SPT. Please justify order.

Thank you for your comment. We corrected the wrong order of the Figs.

* For SPT following chronic stress, how soon does the pref test start after the last restraint. Are animals trained at all prior to starting?

Thank you for your comment. We habituated mice to sucrose from the day after the last restraint. To avoid confusion, we modified the scheme in Figure 2.

Minor Concerns:
* Line 62-64. Sentence states that CIS is used in both acute and chronic stress studies. It would seem by definition that CIS cannot be used for acute stress studies. Perhaps the authors mean that "Restraint stress" can be used both acutely and chronically.

Thank you for your comment. The word 'acute' is wrong. The word was deleted in the introduction.

* Please add additional detail on how the restraint tube was constructed. If purchased, please include manufacturer etc.

Thank you for your comment. We added pictures of the apparatus in Figure 1.

* Line 86: When you say that the animal could not move please be more specific. Could the animal move it's head side to side? Could it move forwards and backwards?

Thank you for your comment. The description were modified from line 113-115 in page 6.

* Describe the lighting used to get "200 lux" light. Is that lux measured at the restraint area or is it a general 200 lux based on a lightbulb or something?

Thank you for your comment. We adjusted the light intensity to 200 lux with a digital lux meter in the restraint area. So, we modified protocol at line 143 in page 7.

* Include in methods or procedure the importance (or lack of importance) on the time of day selected.

Thank you for your comment. We tried to minimize the pain of the mice for animal welfare. 2 hours a day for 15 days is the condition that can cause depressive behaviors with the least time in the chronic restraint stress model used so far. We could not add it to the method and procedure according to the editor's comment, but we added the explanation in Introduction at line 85-88 in page 4-5.

* Line 203: What methods can be used to limit tail climbing in C57 mice?

In our experimental conditions, the behavior of climbing on the tail was not frequently observed. We prevented them with acrylic bar while the mouse climbing their tail. And easily purchase or make the climbstopper to prevent tail climbing.

Reviewer #2:

Manuscript Summary:
The article entitled "A chronic immobilization stress protocol for inducing depression like behavior in mice" submitted by Son et al has nothing new to report. Moreover, the way article is written seems not appropriate for a research article. Following are my concerns, based upon which I do not recommend the article for publication

Major Concerns:

Introduction: should introduce why there is need of new animal models. Authors introduce inadequate treatment of depression and need of newer antidepressant.

Thank you for your valuable comments. We use chronic immobilization stress model to investigate the role of mPFC glutamate/glutamine cycle between glutamatergic neuron and astrocyte. The first draft of this article had many deficiencies, so many parts are supplemented based on reviewer's comments. However, we did not intend to introduce newer antidepressants and treatments, but we wanted to introduce a standardized and simplified experimental method for studying depression. And the scope of JoVE is not a suggestion of the new method but is a suggestion includes novel techniques, innovative applications of existing techniques, and gold standard protocols in the physical and life sciences. Therefore, we presented the need for a newly simplified animal model for the depression research from line 82 to line 85 in page 4 and also added new Fig 1 and 3 to explain detailed procedure of CIS induction and recovery effect of glutamine supplementation after CIS.

Methods No new method is introduced. They state for a chronic immobilization stress protocol but in the method they use restraint stress. The method is not adequately described. At places there are only headings and no description.

As explained above, the scope of JoVE is not a suggestion of the new method but is a suggestion includes novel techniques, innovative applications of existing techniques, and gold standard protocols in the physical and life sciences. Therefore, we focused on to set an innovative applications of existing techniques and standardized, simplified experimental protocol for studying depression. The missing parts of the technique description were corrected and expanded throughout the manuscript and newly added figures.

Results: There are a number of reports that predictable stress produces adaptation to stress. There are no signs of adaptation in their model. To consider chronic immobilization or restraint a model of depression they should include data after single immobilization or restraint stress. Also to justify it as a model of depression they should show effectiveness of antidepressants on the model.

Thank you for your comments. We modified the insufficient of description in the overall manuscript. As a stress marker, changes of corticosterone levels was added in Fig 2. Fig 3 showed recovery effect on glutamine supplementation.

Discussion: the discussion should include comparison of their model with the existing model to show advantages over the current models.

Thank you for your comments. We suggest advantages of CIS and considerations in the Introduction (from line 81 to 83 in page 4) and in the discussion (from line 296 to 298 in page 14 and from line 307 to 310 in page 15).

Minor Concerns:
General: In general the article provides no new or publishable data.

We modified the insufficient of description in the overall manuscript and added new figs.

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