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TITLE:

Burn Injury-induced Pain and Depression-Like Behavior in Mice

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SUMMARY:

A transient scald injury (65 °C ± 0.5 °C, 3 s) of one hind paw decreases the threshold (g) to von Frey filament stimulation of the ipsilateral side and alters gait pattern. Besides, burn injury induces depression-like behavior in the forced swimming test.

ABSTRACT:

Scalding water is the most common cause of burn injury in both elderly and young populations. It is one of the major clinical challenges because of the high mortality and sequelae in low- and middle-income countries. Burns frequently induce intense spontaneous pain and persistent allodynia as well as life-threatening problem. More importantly, excessive pain is often accompanied by depression, which may significantly decrease the quality of life. This article shows how to develop an animal model for the study of burn-induced pain and depression-like behavior. After anesthesia, burn injury was induced by dipping one hind paw of the mouse into hot water (65 °C ± 0.5 °C) for 3 s. The von Frey test and automated gait analysis were performed every 2 days after burn injury. In addition, depression-like behavior was examined using the forced swimming test, and the rota-rod test was performed to differentiate the abnormal motor function after burn injury. The main purpose of this study is to describe the development of an animal model for the study of burn injury-induced pain and depression-like behavior in mice.

INTRODUCTION:

Tissue damage, such as burn and trauma, is generally associated with the co-occurrence of acute pain. Burn injuries and trauma-related symptoms are an estimated 1,80,000 deaths every year are caused by burns—the vast majority occur in low- and middle-income countries from different types of burns[1]. According to a worldwide report, burns are common in children and account for about 40%–60% of hospitalized patients[2,3]. These specific injuries are even more serious as they can occur in everyday life, such as boiling or bathing water[4,5]. Although acute pain can be resolved spontaneously after recovery from tissue damage in most cases, it may be possible to become chronic due to abnormal changes in the nervous system[6,7].

Recently, it has been suggested that acute pain can induce a depressed mood, and chronic pain can cause anxiety and depression[8–11]. The coexistence of pain and depression makes it more difficult to treat the patient. Depression also tends to increase pain sensitivity, which is likely to induce more intense depression and pain[12]. Complications of pain and depression are shown in animal models of peripheral inflammation[13–16]. The detailed mechanisms underlying pain-induced depression are not well known until now[17]. Thus, it is necessary to develop more effective treatments for burns to alleviate the side effects and symptoms.

Thus, the present study was designed to develop an animal model to study burn injury-induced acute pain and depression-like behavior in mice. For this, burn injury-related abnormal tactile sensitivity, altered gait pattern, and depression-like behavior were measured. In addition, this study attempts to validate the model using NSAIDs.

PROTOCOL:

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Chungnam National University in South Korea, and then conducted based on the ethical guidelines of the International Association for the Study of Pain[18].

1. Induction of scalding burn injury on the hind paw

1.1. House the male ICR mice weighing 20–25 g in light and temperature-controlled room (12/12 h light-dark cycle, 22.5 °C ± 2.5 °C) with a humidity of 40%–60%.

1.2. Allow the animal free access to food and water, and acclimatize for at least 1 week before starting the experiment.

NOTE: All the animals were group-housed to exclude variables such as isolation stress.

1.3. Assign the mice randomly to the experimental or control group and conduct blind experiments using animal numbers as codes.

1.4. On the day of burn induction, anesthetize the mouse by intraperitoneal (i.p.) injection of

300 µL of alfaxalone at a dose of 10 mg/kg. Wear a surgical gown, gloves, and mask while performing the burn induction.

1.5. After deeply anesthetizing the mouse, disinfect around the right hind paw with 70% ethanol.

NOTE: Check the lack of response to pinch stimulation applied to the hind toes or tail to confirm the state of deep anesthesia.

1.6. Apply an ophthalmic ointment to the eyes to prevent corneal drying after induction of anesthesia.

1.7. Immerse the right hind paw of the deeply anesthetized mouse in hot water at 65 °C ± 0.5 °C for 3 s. Make a mark on the ankle of each mouse before immersing the hind paw in hot water to maintain consistency in the burned area.

1.8. After induction of burns, bring the mice in a clean home cage and place them on a heating pad until the animals recover from anesthesia.

NOTE: The analgesic agent, acetaminophen (200 mg/kg), was administered intraperitoneally once daily for 7 days starting from the day of burn injury (Only Burn + Acetaminophen group). The Burn group was treated with saline as vehicle control. The experiment was performed according to the method described in a previous study[4].

2. Measurement of mechanical allodynia

2.1. Bring the mice to the behavioral testing room and let them acclimatize at least for 30 min prior to the test. Wear a surgical gown, gloves, and mask while performing the test.

2.2. Place the mice into a square box (diameter: 13 cm, height: 12 cm) on a metal mesh floor (mesh size: 0.7 cm x 0.7 cm) and let them acclimatize for at least 30 min.

2.3. Assess the mechanical threshold of the hind paw using the ascending stimulus method[19,20].

2.4. Gently poke a series of von Frey filaments with 5–8 s intervals to stimulate the hind plantar. Obtain the baseline values on the day before burn induction.

NOTE: The 0.16–1.2 g von Frey filaments were used in the test to measure the paw withdrawal threshold in all animals, respectively. The paw withdrawal response test was started with the lowest bending force of von Frey filament (0.16 g in this protocol). If there was no response, then a filament with the next bending force was applied.

2.5. Perform five trials to evaluate mechanical thresholds for each ipsilateral (injured) hind

paw.

NOTE: The bending force of von Frey filament that produces response more than three times of the five trials in each animal was expressed as paw withdrawal threshold (PWT, g). Mechanical thresholds were measured a day before and at 1, 3, 5, and 7 days after burn injury. Analgesic effect was assessed 1 h after administration of the acetaminophen in the animal.

3. Automated gait analysis

3.1. Acclimate the mice in the gait analysis system once daily for 10–15 min from 5 days before the burn injury. Wear a surgical gown, gloves, and mask while performing gait analysis.

3.2. On the day of the test, bring the mice to the behavioral test room and acclimatize them for at least 30 min before the test.

NOTE: Perform acclimation and gait analysis tests in a dark environment. Set the conditions of the program menu as follows.

3.3.1. After running the program, click on the **Create New Experiment** menu to designate the folder to save the data.

3.3.2. After designation, set the maximum running time to 5 s and maximum allowed speed variations to 50%.

3.3.3. Select a registered camera and set the walkway length to 30 cm in the **Setup** tab of the program.

3.3.4. On the **Acquire** tab of the program menu, select **Open Acquisition**.

3.3.5. Based on the status messages, click on the **Snap Background** button to acquire a background image of an empty walkway.

3.4. Click on the **Start Acquisition** button, and then place the mouse at the entrance of the left-right traversable walkway. The recording will automatically start following the free movement of the mouse.

NOTE: If the animal's gait has been successfully recorded and all footsteps have been detected, it will be marked as Compliant Run with a green icon. If the software does not detect any footsteps, a red icon is displayed, in which case it is recommended to perform the recording again. The authors recommend collecting and analyzing at least five successful compliant runs performed with similar running speeds.

3.5. On the **Acquire** tab of the program menu, select **Classify Runs**.

NOTE: After selecting the data obtained from the successful compliance run above, go to the video analysis screen where the gait patterns of the mice were recorded.

3.6. Select the run to be analyzed and click on the **Auto Classify** button.

3.7. After performing automatic classification, remove nose, genital recognition, and the misrecognition of paws to junk data in each run, and then analyze the data.

NOTE: All statistical parameters are automatically analyzed and saved in the program, and raw data values can be found in the experimenter's analysis menu. Automatic gait analysis was performed before and at 1, 3, 5, and 7 days after burn injury. The evaluation was performed 30 min after acetaminophen administration in the Burn + Acetaminophen group and 30 min after saline treatment in the Burn group. This experiment was performed according to the method described in the previous studies[4,21,22].

4. Measurement of depression-like behavior

NOTE: Despair-based behavior, immobility time in the water was measured by the forced swimming test.

4.1. Bring the mice to the behavioral test room and acclimatize them for at least 30 min before the test. Wear a surgical gown, gloves, and mask while performing the forced swimming test.

4.2. Put the mouse into a clear plexiglass cylinder (10 cm x 25 cm) containing 15 cm of water (25 °C ± 0.5 °C) for 15 min.

4.3. After 24 h, put the mouse into the cylinder of the same conditions and measure the immobility time.

NOTE: Immobility time was measured for 5 min of test time, and the time whenever mice stopped climbing or swimming and just floated to keep their head above the water surface was recorded. The forced swimming test was performed on day 7 after the burn injury. The evaluation was performed 1 h after acetaminophen administration in the Burn + Acetaminophen group, and 1 h after saline treatment in the Burn group. The experiment was performed according to the method described in previous studies[23,24].

5. Measurement of normal motor function

NOTE: The rota-rod test was performed to differentiate the abnormal motor function after burn injury.

5.1. Bring the mice to the behavioral test room and acclimatize them for at least 30 min before the test. Wear a surgical gown, gloves, and mask while performing the forced swimming test.

5.2. Place the animals on a rolling cylindrical platform (5.7 cm wide; 3 cm diameter) suspended 16 cm above the bottom of the apparatus.

5.3. Allow each animal to train once a day on a rota-rod for at least 5 days prior to induction of burn injury.

5.4. Perform the rota-rod test every 20 min for 2 h after drug administration. Set the cut-off time to 2 min.

5.5. Measure the duration of time the mouse runs on a rotating rod at the constant speed of 15 revolutions per minute without falling.

NOTE: The rota-rod test was performed 7 days after the induction of burn injury. The evaluation was performed immediately after acetaminophen administration in the Burn + Acetaminophen group and after saline treatment in the Burn group. Alfaxalone was used as a positive control for experimentally treated drugs in this test. During the rotarod test, the duration of time the mouse runs on the rotating rod without falling is measured. The experiment was performed according to the method described in previous studies[22,25].

REPRESENTATIVE RESULTS:

In order to minimize animal suffering and reduce the number of animals used per the Three Rs (Replacement, Reduction, and Refinement) guidelines, this study was designed with the minimum number of animals for the collection of significant data established through preliminary experiment. In this study, behavioral experiments were independently conducted twice as follows. The gait analysis, mechanical allodynia, and depression-like behavior tests were conducted with Control (n = 5), Burn (n = 7; vehicle control; saline), and Burn + Acetaminophen (n = 7) groups. In the rota-rod test, Control (n = 3), Burn (n = 4; vehicle control; saline), Burn + Acetaminophen (n = 4), Positive control (n = 4; Alfaxalone) groups were designed. Alfaxalone is a type of neuroactive steroid and anesthetic that is currently used in veterinary medicine as an injectable general anesthetic inducer. In this study, alfaxalone was used in animal anesthesia for burn induction and used as a positive control drug for motor impairments in the rota-rod test.

Data were expressed as mean ± S.E.M. In addition, experimental data obtained at different times were analyzed independently. Pain behavioral responses were calculated as the area under the curve (AUC). Two-way repeated measures ANOVA was performed to determine differences in the data from mechanical allodynia test, gait analysis, and rota-rod test over time. Dunnett's test was used for Post-hoc analysis to determine the *P*-value among the experimental groups. Immobility time data obtained from the forced swimming test was analyzed using the student's *t*-test to compare the two means. *P*-values less than 0.05 were considered significant. GraphPad Prism 6.0 software was used to analyze this statistical validity. All statistical analysis procedures were performed blindly in relation to the experimental conditions. A figure illustrating the burn injury-induced tissue damage is shown in **Supplementary Figure 1**.

Time-course changes in the paw withdrawal threshold (PWT, g) after burn injury are shown in

Figure 1. The PWT (g) of burn injury-induced mice was decreased 1 day after burn-induction and sustained for 7 days compared with that of the control group. Acetaminophen administration (200 mg/kg, i.p., once daily for 7 days starting from the day of burn-induction) significantly reduced the burn-induced decrease in PWT (**Figure 1A**, ** $p < 0.01$ versus Burn group). In addition, the AUC analysis (for 7 days) showed that acetaminophen administration significantly decreased the burn injury-induced mechanical allodynia (**Figure 1B**, *** $p < 0.001$ versus Control group, ** $p < 0.01$ versus Burn group).

Changes in the hind paw print area after burn injury over time are shown in **Figure 2**. Burn injury significantly reduced the ipsilateral hind paw print area from the day after induction and persisted for 7 days. The hind paw print area was significantly improved by administering acetaminophen (200 mg/kg, i.p., once daily for 7 days starting from the day of burn-induction) compared with the vehicle-treated group (**Figure 2A**,**B**, * $p < 0.05$ and ** $p < 0.01$ versus Burn group).

Time course changes in a single stance after burn injury are shown in **Figure 3**. Burn injury reduced the single stance (%) of the ipsilateral hind paw 1 day after burn-induction, and this reduction was maintained for 7 days. The hind paw single stance was improved by acetaminophen administration (200 mg/kg, i.p., once daily for 7 days starting from the day of burn-induction) compared with the vehicle-treated group (**Figure 3A,B**, * $p < 0.05$ versus Burn group).

Changes in the immobility time obtained from the forced swimming test are shown in **Figure 4**. Immobility times of the burn injury-induced mice were increased 7 days after burn induction as compared with that of the control group. In burn injury-induced mice, acetaminophen administration (200 mg/kg, i.p., once daily for 7 days starting from the day of burn induction) significantly reduced the burn injury-induced increase in immobility time (** $p < 0.01$ and *** $p < 0.001$ versus Burn group).

The normal motor function was assessed based on the changes in running time on the rota-rod, as shown in **Figure 5**. Running times of the burn injury-induced mice did not change at 7 days after burn induction compared with that of the control group. By contrast, the running times of alfaxalone-treated mice (positive control) were significantly decreased during about 60 min. This result indicates that a burn injury used in this study does not cause motor impairment (*** $p < 0.001$ versus Burn group).

FIGURE AND TABLE LEGENDS:

Figure 1: Mechanical allodynia assessed by von Frey test in burn injury-induced mice. (**A**) The paw withdrawal threshold (PWT, g) in the ipsilateral hind paw of mice was decreased 1 day after burn injury and sustained for 7 days as compared with the Control group. Acetaminophen administration (200 mg/kg, i.p., once daily for 7 days starting from the day of burn induction) significantly reduced the burn injury-induced mechanical allodynia. (**B**) The PWT was analyzed as the area under the curve (AUC). Arrows indicate the day of drug administration. *** $p < 0.001$ versus Control group, ** $p < 0.01$ versus Burn group. Two-way repeated measures ANOVA was

performed to determine overall effects in the time-course of the von Frey test. Post-hoc analysis was performed using Dunnett's test in order to determine the *P*-value.

Figure 2: Paw print area obtained from automated gait analysis in burn injury-induced mice. (**A**) Representative images of both the ipsilateral and contralateral hind paws of mice were captured by the gait analysis software. The contact size of the paw is reduced after burn injury compared to that of the Control group. This reduction was partially recovered by administering acetaminophen (200 mg/kg, i.p., once daily for 7 days starting from the day of burn induction). White rectangles indicate the hind paws analyzed by the software, (**B**) a graph shows the time course changes in the paw print area (%). Data are calculated as the percent of changes in the print area between the ipsilateral (right) and contralateral (left) hind paws (e.g., the value of 50% indicates the same paw print areas in the right and left hind paws). Arrows indicate the day of drug administration. * $p < 0.05$ and ** $p < 0.01$ versus Burn group. Two-way repeated measures ANOVA was performed to determine overall effects in the time-course of the print area on gait analysis. Post-hoc analysis was performed using Dunnett's test in order to determine the *P*-value.

Figure 3: Single stance obtained from automated gait analysis in burn injury-induced mice. (**A**) Representative images of a single stance were captured by the gait analysis software. Different colors indicated the stance of each paw: blue, right front paw; pink, right hind paw; yellow, left front paw; green, left hind paw. The single stance of the ipsilateral hind paw was shortened after burn injury. This change was partially recovered by administering acetaminophen (200 mg/kg, i.p., once daily for 7 days starting from the day of burn induction). (**B**) A graph shows time-course changes in the single stance (%). Data are summarized as a line graph after calculating the percent of changes in single stance between the ipsilateral (right) and contralateral (left) hind paws (e.g., the value of 50% indicates the same single stance in the right and left hind paws). Arrows indicate the day of drug administration. * $p < 0.05$ versus Burn group. Two-way repeated measures ANOVA was performed to determine overall effects in the time-course of single stance on gait analysis. Post-hoc analysis was performed using Dunnett's test in order to determine the *P*-value.

Figure 4: The immobility time of the forced swimming test in burn injury-induced mice. Immobility times of the burn injury-induced mice were increased 7 days after burn induction as compared with that of the Control group. In burn injury-induced mice, acetaminophen administration (200 mg/kg, i.p., once daily for 7 days starting from the day of burn induction) significantly alleviated burn injury-induced enhanced immobility time. *** $p < 0.001$ versus Control group, ** $p < 0.01$ versus Burn group. One-way repeated measures ANOVA was performed to determine overall effects in the time-course of the forced swimming test. Post-hoc analysis was performed using Dunnett's test in order to determine the *P*-value.

Figure 5: The normal motor function assessment based on the changes in running time of the rota-rod test in burn injury-induced mice. There was no change in running times of the burn injury-induced mice at 7 days after burn induction as compared with those of Control and acetaminophen-treated burn injury groups. However, the running time of alfaxalone-treated mice (Positive control) was significantly decreased to ~60 s. This result indicates that a burn injury used in this study does not cause motor impairment. *** $p < 0.001$ versus Burn group. Two-way

repeated measures ANOVA was performed to determine overall effects in the time-course of the rota-rod test. Post-hoc analysis was performed using Dunnett's test in order to determine the *P*-value.

Supplementary Figure 1: Changes in tissue damage over time after induction of burns. After the scalding burn induction, significant tissue damage was observed, which increased gradually over time. In this study, Acetaminophen, used as a positive control drug, has shown a protective effect on tissue damages.

DISCUSSION:

The scalding burn is a kind of thermal burn that is caused by heated liquids. It has been suggested that first- or second-degree burns occur in most cases, but long-term contact with heat sources can cause third-degree burns[26]. In the present study, burn injury was induced by exposing the right hind paw of mice into hot water at 65 °C for 3 s[4,26]. Tissue damage was detected in the burn-injured paw, which shows common symptoms of burns such as redness, peeling of the skin, and swelling (**Supplementary Figure 1**)[4].

Mechanical allodynia measurement is a commonly used pain response identification method in animal pain models and was measured using von-Frey filaments in this study. The ascending stimulus method with the von Frey filaments is used to determine the mechanical threshold required to induce an animal's paw withdrawal response[19,20]. The experiment started with the filament with the least stimulus. The bending force of the filament responding to a set number (three times in this protocol) was obtained as a paw withdrawal threshold value.

Gait analysis of rodents during free walking is used to study Parkinson's disease or limb movements and position changes in sensory-motor impairment models, including spinal cord injury and stroke[27,28]. The gait analysis system automatically analyzes various gait parameters, including paw intensity, paw print, stance phase, etc. Alterations of parameters that the gait analysis system can analyze can be used as pain-related indicators in the gait analysis of pain animal models. Therefore, gait analysis may be used as an experimental method to non-invasively quantify spontaneous pain in animal models[4,21,22]. Based on previous findings that gait parameters were decreased on the pain-induced side in animal models of pain[21,22], this presented protocol quantified each gait parameter as the ratio of the burn-induced ipsilateral and contralateral side. In this protocol, paw print area and single stance data were converted into the rate of changes between ipsilateral (injured) and contralateral (non-injured) hind paws. The value of 50% means that the paw print size and the time to reach the floor are the same in both the ipsilateral and contralateral, while the value less than 50% indicates that these parameters are decreased in the burn-induced ipsilateral hind paw. The percentage changes between the ipsilateral and contralateral hind paws were used for obtaining all the data (i.e., normal mice showed ~50%, which means that the ipsilateral: contralateral ratio was 50:50). In normal animals, the parameters related to each hind limb appear the same on both sides when walking freely. However, the analysis of this protocol is focused on the fact that the parameters on the ipsilateral side decrease after pain induction. In addition, there is individual variation in each animal; accurate data may not be obtained when the raw data is analyzed as is. Therefore, each gait

parameter value was converted into a ratio to obtain more accurate results during analysis. The present study has shown that the print area size and the single stance time of the ipsilateral hind paw were reduced after burn injury, and this reduction was restored by repeated intraperitoneal acetaminophen administration. These changes coincided with a similar pattern of time-course changes in pain behaviors after burn injury and drug administration.

Although controversial, the forced swim test is the most commonly used method to study the behavior of depressed rodents. The animals attempt to escape from the container full of water but eventually do not move, inducing despair[29]. However, it is argued that immobility is difficult to evaluate as a measure of depression because this test is associated with endurance as well as feelings of despair. To support the results of the forced swimming experiment, other methods of evaluating depression, such as the tail suspension test, novelty-suppressed feeding test, and sucrose consumption test, may be considered[30,31]. In the present study, immobilization time was increased after burn injury, and this increase was restored by acetaminophen administration.

The protocols of this study were designed to establish a model of acute pain accompanying depression-like behavior after burn injury. The depression-like behavior in this study may be the secondary effects of physical impairment and changes in thermal sensitivity after burn injury[15,32,33]. The results could suggest that mice with induced acute pain after burn injury exhibited depression-like behavior. It has been shown to improve pain response and consequent depression-like behaviors by experimentally treated drugs.

The rota-rod test is a performance test based on a rotational load that is generally forcibly applied to rodents with athletic activity. The test measures parameters such as running time and endurance. Some of the test features include, among others, the effects of an experimental drug or the balance of subjects in the neuropathic pain model, grip strength, and motor coordination assessment[22,25,34]. As shown in the results of this study, there was no change in the rota-rod running times following burn injury or acetaminophen treatment compared with that of the control group.

This study demonstrates the development of an animal model for studying burn injury-induced pain and depression-like behavior in mice. In this regard, this study has shown that scalding burn injuries induced mechanical allodynia, alterations of gait parameters, and depression-like behavior such as immobility time. This model is suitable for research on the various aspects and outcomes of burn pain and its treatment and is expected to bring important information to this research field.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

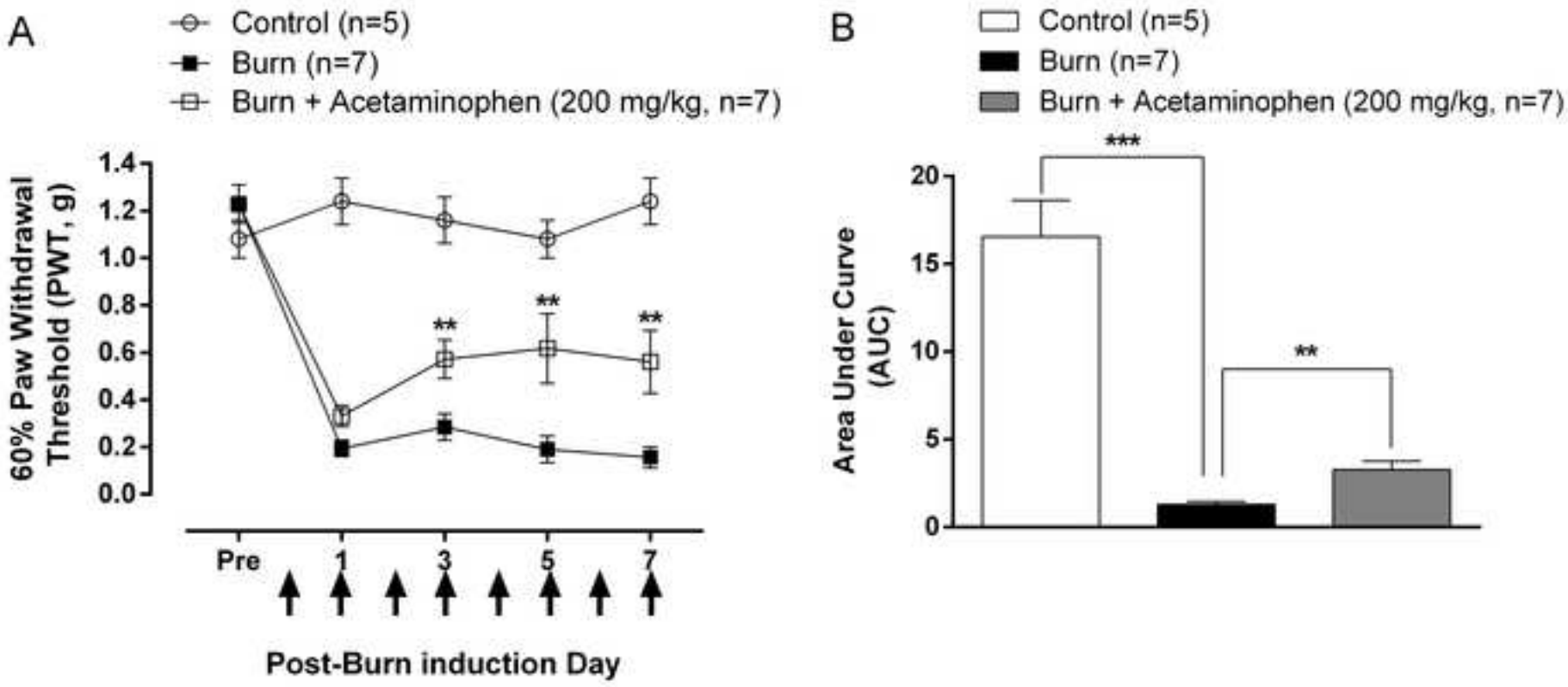
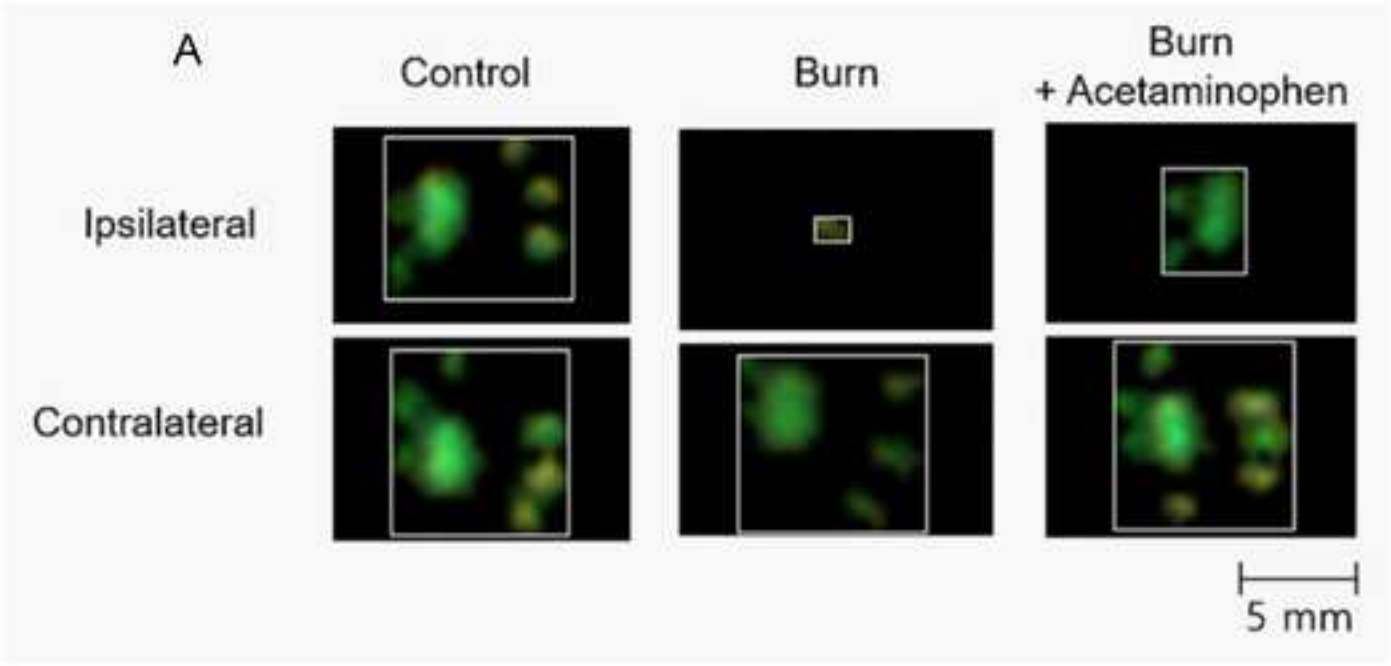


Figure 2



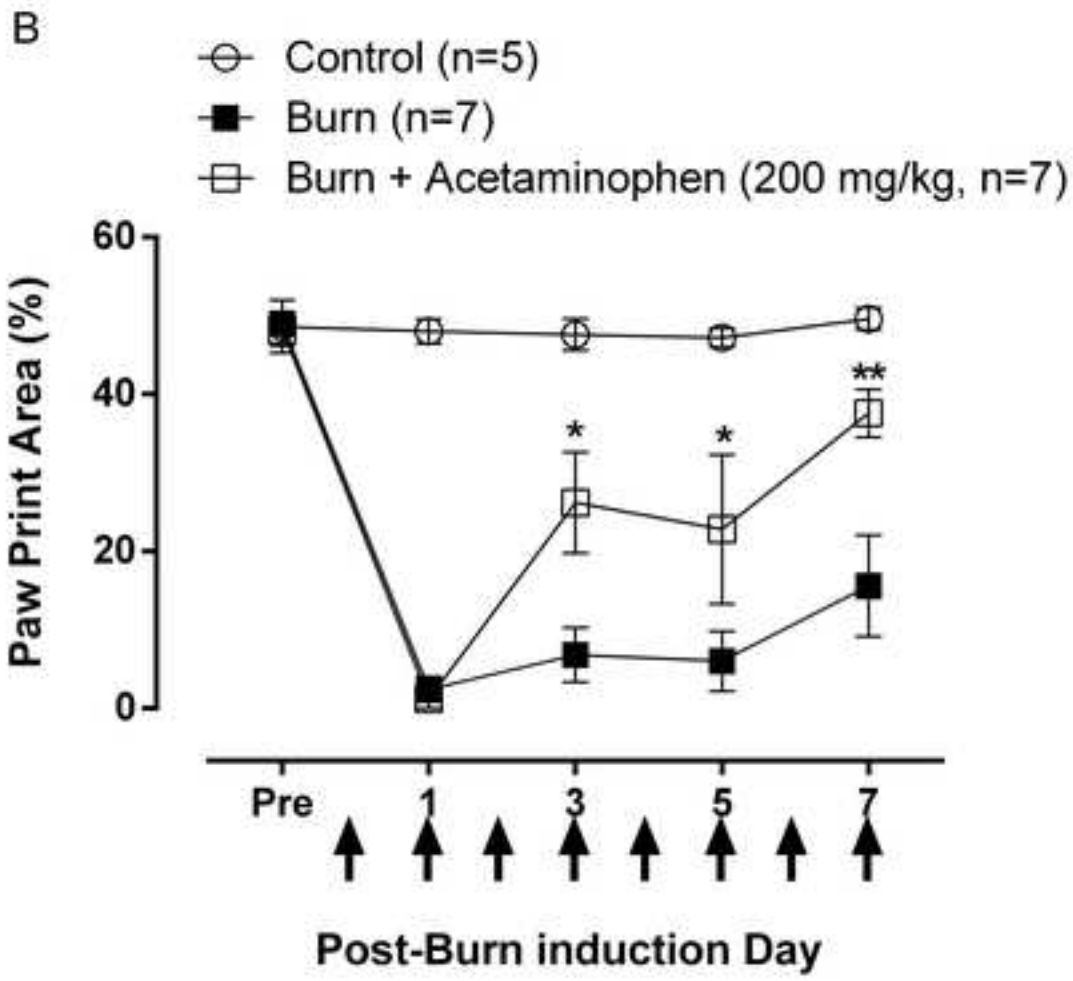


Figure 3

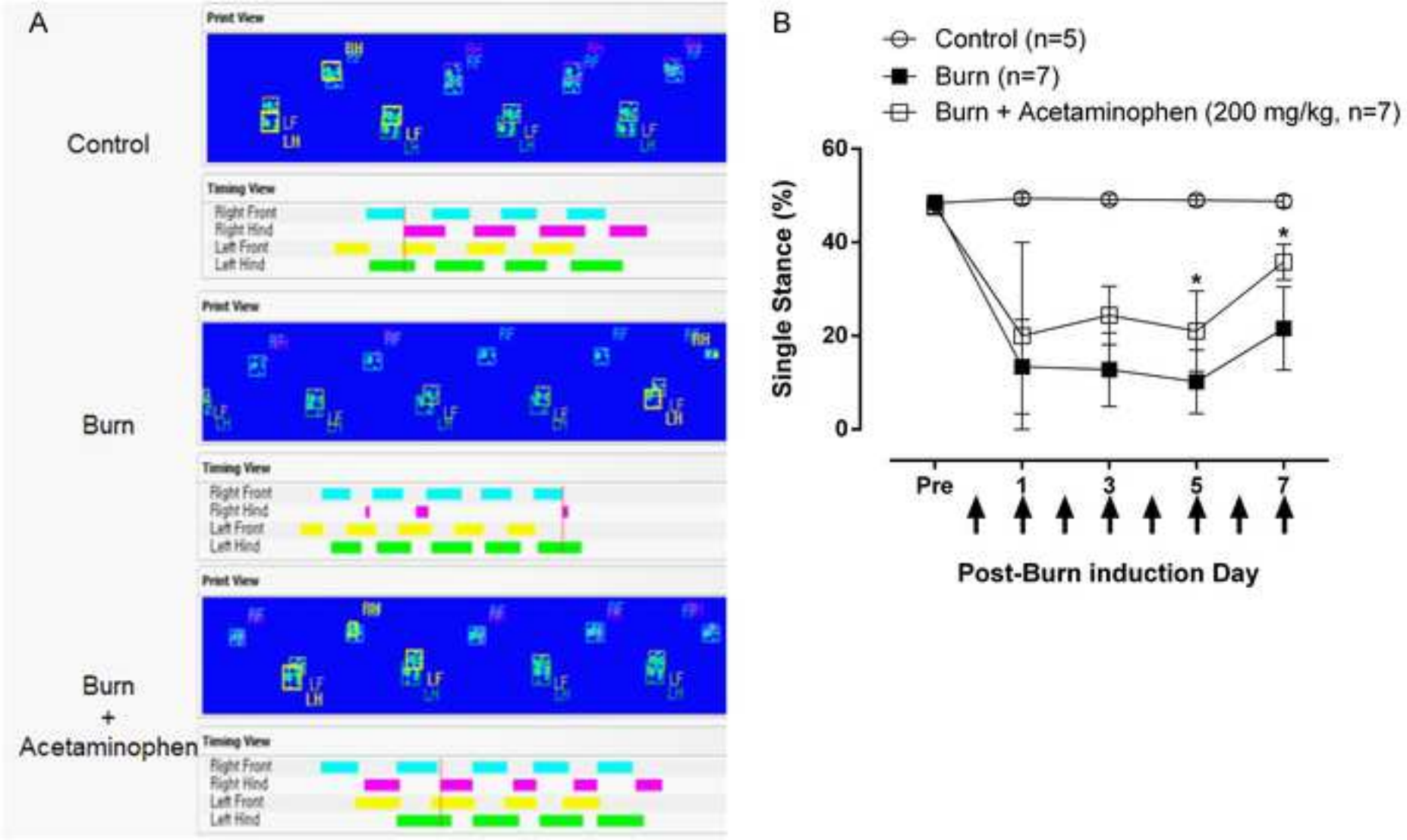


Figure 4

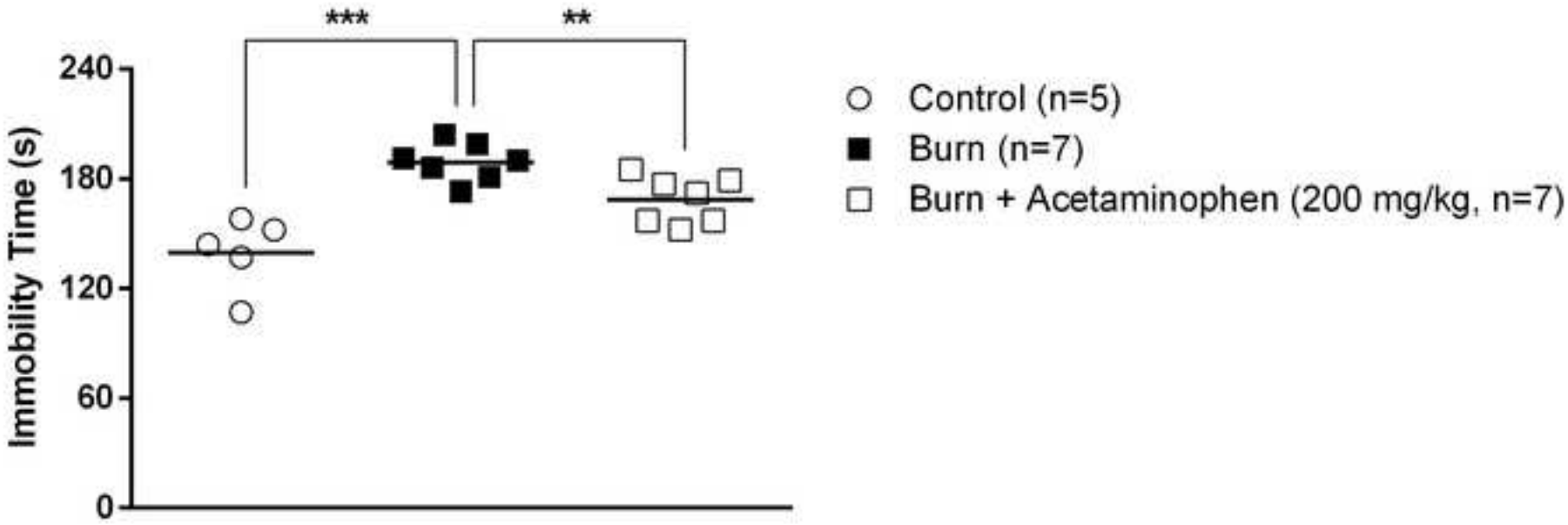
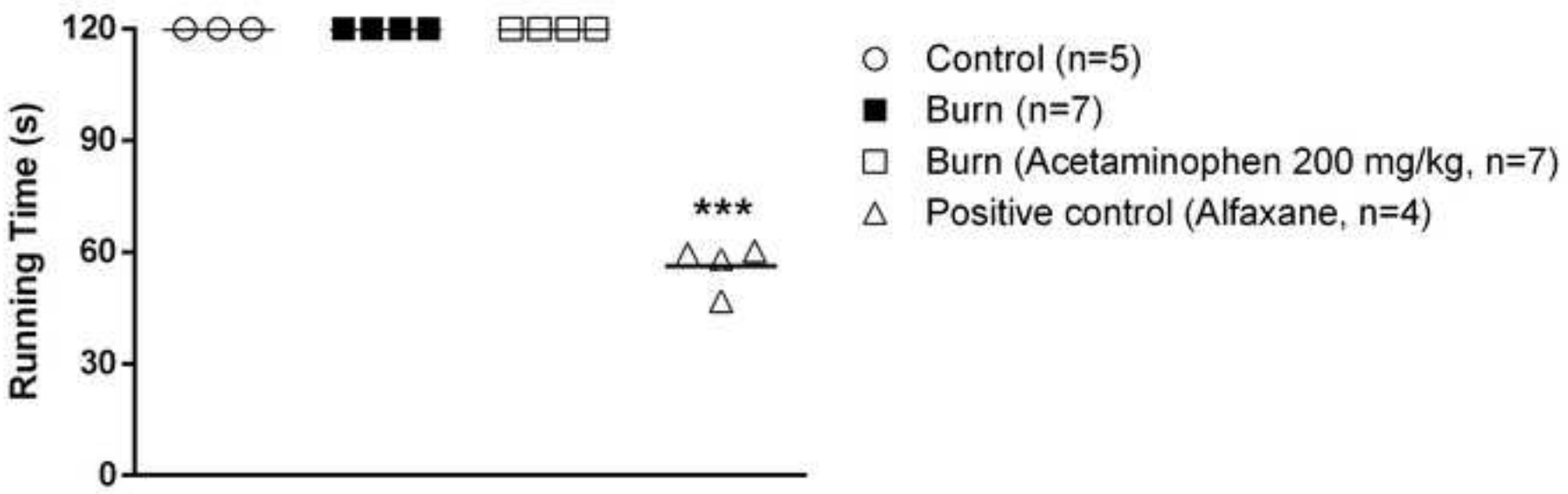


Figure 5



Date: Sep. 15, 2021

Dr. Amit Krishnan, Ph.D.
Review Editor
JoVE
amit.krishnan@jove.com

Dear Editorial Office of JoVE

Please find an electronic copy of our secondly revised manuscript entitled, “Burn Injury-induced Pain and Depression-Like Behavior in Mice” (Manuscript Number: JoVE62817R2) which we are resubmitting for consideration of publication in the JoVE. We have reorganized the manuscript and attempted to address all reviewers’ criticisms and concerns.

If we can, we would like to have a new co-author because of her great effort on the second revision. The author's information is like below;

Miae Lee
Department of Physiology and Medical Science, College of Medicine and Brain Research Institute, Chungnam National University, Daejeon 35015, South Korea (miae@cnu.ac.kr)

We hope that you will now find this manuscript suitable for publication. We look forward to your comments on the revised manuscript and we would be happy to make additional changes as suggested by you or the reviewers to further improve the manuscript if necessary.

Thank you for considering our paper for possible publication in JoVE.

Sincerely,

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- **Editorial comments and responses**

1. Please note that the manuscript has been formatted to fit the journal standard. Some comments to be addressed are included in the manuscript file itself. Please review and revise accordingly.

Response: The entire manuscript has been revised according to the editor's comments. Revisions are indicated in red text.

2. Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Following the editor's comment, we have marked the contents of the protocol that requires visualization. The contents were highlighted in bigger font size and bold text.

3. Figure 4/5: Please revise the Y-axis unit to “s” instead of “sec”.

Response: Following the editor's comment, the Y-axis unit was changed to "s" in figures 4 and 5.

4. Please revise the Table of Materials to contain all the essentials (consumables, chemicals, reagents, equipment, etc.) used in this study.

Response: Following the editor's comment, the table of materials was revised.

- **Reviewer’s comments and responses**

Reviewer #1:

Manuscript Summary: Successfully revised.

Response: Thank you for your comment.

Reviewer #2:

Major Concerns:

The manuscript has improved from the last revision!

I though continue to point out, that I find it counter-intuitive that there's an introduction about chronic pain and emotional comorbidities, stating that the purpose of the study is to develop a model for this, while the measure of "depressive-like behaviour" is at day 7, - which is not at all at a chronic stage.. And I still disagree with the use of forced swim test when assessing a gait- and sensory-impaired model. The forced swim test is already under a lot of critical debate as a measure of depressive-like behaviour within the scientific community, and when then being potentially affected by the induction of cold-sensitivity and functional impairment from the burn-injury, rather than only emotional impact, I find it more problematic. I therefore urge you to remove the forced swim test from the JOVE-production/manuscript, and if anything then replace it with an alternative test of depressive-like behaviour, which is not as controversial or potentially affected by sensory or functional impacts of the injury. Alternatively, then the discussion of these limitations needs to be clearer than what had been added so far.

In my opinion, then the sensory- and gait-related outcomes, and validation using a reference analgesic compound, remains more than enough to characterize and present this model on it's own!

Response: Thank you for your great advice. Following the reviewer's advice, the introduction part, which mentioned the relationship between chronic pain and depression, was modified to fit the context of this study.

“Recently, it has been suggested that acute pain can induce to depressed mood and chronic pain cause anxiety and depression[8,9,10,11]. The coexistence of pain and depression makes it more difficult to treat the patient. Depression also tends to increase pain sensitivity, which is likely to induce more intense depression and pain12. Complications of pain and depression are shown in animal models of peripheral inflammation[13,14,15,16]. The detailed mechanisms underlying pain-induced depression are not well known until now[17]. Thus, it is necessary to develop more effective treatments for burns to alleviate the side effects and symptoms.

Thus, the present study was designed to develop an animal model for the study of burn injury-induced acute pain and depression-like behavior in mice. For this, burn injury-related abnormal tactile sensitivity, altered gait pattern and depression-like behavior were measured. In addition, this study attempts to validate the model using NSAIDs.”
(Line 56~67)

Reference:

8 Michaelides A, Zis P. Depression, anxiety and acute pain: links and management challenges. Postgraduate medicine. Sep;131(7):438-444, (2019).

9 Doan, L., Manders, T., Wang, J. Neuroplasticity underlying the comorbidity of pain and depression. Neural plasticity, 2015, 504691, (2015).

13 Deng, Y. T., Zhao, M. G., Xu, T. J. Gentiopicroside abrogates lipopolysaccharide-induced depressive-like behavior in mice through tryptophan-degrading pathway. Metabolic brain disease, 33(5), 1413–1420, (2018).

14 Zhang, G. F., Wang, J. Acute single dose of ketamine relieves mechanical allodynia and consequent depression-like behaviors in a rat model. Neuroscience letters, 631, 7–12, (2016).

Minor Concerns: Line 74; it still remains unclear how it was determined, how many animals were necessary for "meaningful data could be collected"..? Power-calculation or experience from previous studies using the same assays..? This protocol is supposed to teach scientists how to perform this line of research, and therefore it is necessary to know

how you have determined how many animals to include in each group. It's for instance unclear why some groups contained as little as 3 animals, others 4 or 5, and some 7..? Why were control-groups generally smaller than other groups..? Which factors went into this decision-making? The reader needs this information, so that they can understand how to design their own experiments using this protocol.

Response: Thank you for your comment. As commented by a reviewer, we have corrected the sentence in the manuscript as follows.

“In order to minimize animal suffering and reduce the number of animals used in accordance with the Three Rs (Replacement, Reduction, and Refinement) guidelines, this study was designed with the minimum number of animals for which significant data could be collected, established by preliminary experiment.” (Line 73~77)

Line 81-82; so the experiment was repeated 3 times independently..? This statement raises more questions than it answers. Does this mean that the groups mentioned of n=3-7 was all run 3 times (so that all group-sizes should be multiplied with 3), or does this mean that these groups were divided in 3 cohorts to make up the full group-size of n=3-7 ? In that case, did the cohorts include equal numbers of animals from each group, or how was this organized ? Or was the rotarod for instance run as an independent study, and the other studies divided in two..?

Response: Thanks for your comments. Sorry for providing confusing information. As commented by a reviewer, relevant protocol sessions were modified as follows.

“In this study, behavioral experiments were independently conducted twice as follows. The gait analysis, mechanical allodynia, and depression-like behavior tests were conducted with Control (n = 5), Burn (n = 7; vehicle control; saline), and Burn + Acetaminophen (n = 7) groups. In the rota-rod test, Control (n = 3), Burn (n = 4; vehicle control; saline), Burn + Acetaminophen (n = 4), Positive control (n = 4; Alfaxalone) groups were designed.”
(Line 77~81)

Line 88: given continued discussions among scientists in the field, where some choose to single-house their animals for experiments exploring anxio-depressive behaviors after pain-injuries, it is relevant to mention if the animals were housed alone or in groups. Single-housing is a stressor to rodents, and some scientists in the field claim that this is necessary to enhance the anxio-depressive phenotype displayed after injury, - others argues that the added stress of single-housing means that the model is no longer a model of only "pain", but rather "stress+pain", and therefore they choose group-housing. It is therefore necessary to state what was chosen for this protocol for replication-purposes. Was the animals housed alone or in groups ?

Response: Thank you for your comment. As commented by the reviewers, we added NOTE to the relevant protocol session to clarify as follows.

NOTE: All animals were group-housed to exclude variables such as isolation stress.
(Line 94~95)

Line 122-141: Mechanical threshold

It remains unclear how the threshold was reached, and I recommend rewriting this part to explain exactly what was done, as the reader now need to find some pieces of information in section 2.1-2.4, while other details to understand the protocol, are hidden in the "Note", - and those sections doesn't always seem to agree or be aligned.

The referred paper by Pitcher and Chaplan describes the most broadly used calculation of a 50% response threshold, using the up-down method, and next a calculation of the response threshold (50% g threshold = (10 Xf+kδl)/10,000) using the Dixon table. But this does not seem to be what is being shown in the current protocol / was done in the presented experiments..? Therefore you should decide which protocol to follow, and refer only to papers using the same protocol, and explain clearly how to do it.

From the "note" (line 133), I suspect a different approach was followed, with no transformation/calculation, but determination of the threshold as the filament giving at least 3 out of 5 positive responses..? This has been done in other publications, and is also acceptable, but rarely referred to as "60% response threshold", but merely a "paw

withdrawal threshold", in my experience. And if that was the chosen methodology, then other methodology-references using that particular method, should be used, and a comment should be made, that another widely accepted methodology is the one used in the Pitcher-paper (50% response threshold), - which by the way has a very nice and clear description for that methodology. Also keep in mind, that statisticians would argue, that when using the "3/5-approach", the data is not on a continuous scale, and therefore the data should be analyzed/treated as non-parametric, rather than the current analysis (I would recommend consulting a statistician for this part).

Line 126 seems to suggest that mice were tested with a range of 0.4-1.0g, which does not seem to correlate well with the results presented, where the threshold is between 0.2-1.2..? Or does 0.4-1.0g specify the starting point of the first filament ? Then this needs to be specified, and explained why the starting point was not the same for all animals tests.

Line 138 seems to suggest a different range of filaments (0.02-2.0g), which now leaves the reader confused as to what is actually being done, and therefore impossible to adopt the protocol.

Response: Thank you for your comment. During the review process, we recognized that a significant mistake had been made. The mechanical allodynia-related threshold calculation method used in this study was the ascending stimulus method, not the up-down method. In this regard, the contents and references of the protocol session have been modified as follows.

2.3. Assess the mechanical threshold of the hind paw using the ascending stimulus method[19,20]. Stimulate the hind plantar by gently poke a series of von Frey filaments with 5–8 s intervals. Obtain the baseline values on a day before burn induction.

NOTE: The 0.16–1.2 g von Frey filaments were used in the test to measure paw withdrawal threshold in all animals, respectively. The paw withdrawal response test was started with the lowest bending force of von Frey filament (0.16 g in this protocol). If there was no response, then apply a filament with the next bending force.

2.4. Perform five trials to evaluate mechanical thresholds for each ipsilateral (injured) hind paw.

NOTE: The bending force of von Frey filament that produces response more than three times of the five trials in each animal was expressed as paw withdrawal threshold (PWT, g). Mechanical thresholds were measured a day before and at 1, 3, 5, and 7 days after burn injury. Analgesic effect was assessed 1 h after administration of the acetaminophen in the animal. (Line 136~152)

Reference:

19 Deuis, J. R., Dvorakova, L. S. Methods Used to Evaluate Pain Behaviors in Rodents. Frontiers in molecular neuroscience, 10, 284, (2017).

20 Scholz, J., Broom, D. C. Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. The Journal of neuroscience : the official journal of the Society for Neuroscience, 25(32), 7317–7323, (2005).

Discussion,

Line 353-355 needs a reference.

Response: Thank you for your comment. We added reference based on reviewers' advice.

Line 358; as mentioned in the last review, the wording is still problematic. I agree that the described symptoms of swelling, redness etc suggests a "painful injury", but I must emphasize that without asking the "patient"/animal, we cannot be sure (in theory someone could be insensitive to pain, and therefore not perceive pain despite all these inflammatory indicators). Therefore you may refer to all these INFLAMMATORY signs as if they are suggesting a painful injury, or mention that the behavioral tests and observations related to the injured paw is considered "pain-like". But one should not list

"pain" in the end of this list of signs suggesting inflammation, as this is only an assumptions. On the other hand, you may say "….such as redness, peeling of the skin, swelling, and pain-like behavior.."

Response: Thanks for your comments. Following the reviewer's advice, we removed the word "pain" to refer only to signs of inflammation.

Line 360-365, this sections needs to be reworded according to the comments regarding the method-discrepancy, since the method presented in the Note (line 133) does not correspond with the method in the two papers referred to in this section (Pitcher and Chaplan), which report a 50% threshold based on a calculation, not 60% as mentioned in this paragraph. Please clarify this discrepancy in the discussion.

Response: Thank you for your comment. In accordance with the above-mentioned error correction in the protocol session, the sentence has been corrected as follows:

“Mechanical allodynia measurement is one of the commonly used pain response identification methods in animal pain models and was measured using von-Frey filaments in this study. The ascending stimulus method with the von Frey filaments is used to determine the mechanical threshold, which is required to induce an animal's paw withdrawal response[19,20]. The experiment started with the filament with the least stimulus, and the bending force of the filament responding to a set number (3 times in this protocol) was obtained as a paw withdrawal threshold value.” (Line 376~382)

Line 367-369 needs a reference.

Response: Thank you for your comment. We added reference based on reviewers' advice.

“Gait analysis of rodents during free walking is used to study Parkinson's disease or limb movements and position changes in sensory-motor impairment models including spinal cord injury and stroke [27,28].” (Line 384~386)

Reference:

27 Vandeputte, C., Taymans, J. M. Automated quantitative gait analysis in animal models of movement disorders. BMC neuroscience, 11, 92, (2010).

28 Isvoranu, G., Manole, E. Gait Analysis Using Animal Models of Peripheral Nerve and Spinal Cord Injuries. Biomedicines, 9(8), 1050, (2021).

Line 372; "non-irritating"..? Do you mean "non-evoked" ?

Response: Thank you for your comment. To avoid confusion, the content has been modified as follows:

Alterations of parameters that can be analyzed by the gait analysis system can be used as pain-related indicators in the gait analysis of pain animal models. Therefore, gait analysis may be used as an experimental method to non-invasively quantify spontaneous pain in animal models [4,21,22]. (Line 387~390)

Line 388-390; the reason for this test being widely used, is not because animals are also exposed to stress during it! The common rational for using it, has been that it was originally possible to validate the test using some types of anti-depressants. But some continue to argue that this test is not valid to use for assessment of depressive-like behavior, especially not for movement-impaired animals as it's also based on "endurance", and since you show changes in the gait analysis and sensory deficits in VF, this may also affect the results obtained here. I would suggest that this section also include recommendations for alternative tests of depression, to make the reader aware of alternatives available.

Response: Thank you for your good comment. Following the reviewer's advice, we added alternative experimental methods and references for depression as follows.

“Although controversial, the forced swim test is the most commonly used method to study the behavior of depressed rodents. The animals attempt to escape from the container full of water, but eventually do not move, inducing despair [29]. However, it is argued that

immobility is difficult to evaluate as a measure of depression because this test is associated with endurance as well as feelings of despair. To support the results of the forced swimming experiment, other methods of evaluating depression, such as the tail suspension test, novelty-suppressed feeding test, and sucrose consumption test, may be considered [30, 31]." (Line 411~417)

Reference:

30 Yan, H. C., Cao, X. Behavioral animal models of depression. *Neuroscience bulletin*, 26(4), 327–337, (2010).

31 Papp, M., Willner, P. An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. *Psychopharmacology*, 104(2), 255–259, (1991).

Line 401; "this phenomenon " ? Which phenomenon are you referring to ? The rotarod results in the section just before.?

Response: Thank you for your comment. To avoid confusion, the content has been modified as follows:

"The protocols of this study were designed to establish a model of acute pain accompanying depression-like behavior after burn injury. The depressive-like behavior in this study may be secondary effects of physical impairment and changes in thermal sensitivity after burn injury [15,32,33]." (Line 420~423)

Line 401-405 should be moved up to before the discussion of the rotarod-data. Currently the section does not clarify, that other studies suggest longer durations of injury for detection of anxio-depressive like behavior (the referred Yalcin and Seminovicz papers), only a short mention that "it is common in chronic". Also, it is not ALL depressive-like behaviors, that could be secondary effects of the injury (as suggested in line 402), it is specifically the readout from the forced swim, that may be affected by this. And in the light of this, it is not correct to state that "these results show" (line 403), but should be reworded

to "these results could suggest..", as it is not a definitive fact, when there are alternative explanations for the behavior displayed ! I would therefore request a more open discussion of the results, and rewording of this section, so that you first present the potential limitations of the experimental design (timing and assay) in the light of the remaining literature in the field, and then specify that the results of the current experiment, should be considered in the light of this.

Response: Thank you for your comment. Following the reviewer's advice, we changed the order of the paragraphs discussed and revised the sentences as follows:

“The protocols of this study were designed to establish a model of acute pain accompanying depression-like behavior after burn injury. The depressive-like behavior in this study may be secondary effects of physical impairment and changes in thermal sensitivity after burn injury [15,32,33]. The result of present study could suggest that mice with induced acute pain after burn injury exhibited depression-like behavior. It has been shown to improve pain response and consequent depression-like behaviors by experimentally treated drugs.” (Line 420~425)

Figure 4; immobility time. According to the descriptions, the groups were compared using students t-test..? When comparing more than 2 groups, students t-test is not appropriate, but other tests like Bonferroni, Tukey or Dunnett's should be used as post tests following a significant finding in an ANOVA. I find it difficult to understand why a different test was used for this figure/data-set, when Dunnett's post tests were used for the similar AUC-graph in figure 1. The only explanation, I see if that the results were only significant when using a t-test, but not when using Dunnett's, like in the other results… Therefore I suggest being stringent on the choice of statistics, and change to Dunnett's for all graphs/data-sets including more than two groups.

Response: Thank you for your comment. During the review process, we recognized that a mistake had been made. The graph in Figure 4 also performed Dunnett's test to calculate the p-value. We have corrected the word accordingly.

Reviewer #4:

Manuscript Summary:

The authors improved the revised manuscript in a satisfactory manner. I would recommend this paper for publication in JoVE.

Response: Thank you for your comment.

Reviewer #5:

Manuscript Summary:

It appears to be well improved compared to the last submission

Response: Thank you for your comment.

Minor Concerns:

1. Figure 5: No variance is shown in the figure: please provide a scatterplot. Other groups need to see that variance to compare their findings

Response: Thank you for your comment. Following the reviewer's advice, the graph in Figure 5 was changed to a scatter plot.

2. Still no discussion of why the n is so low, or power analysis to show they are sufficient.

Response: Thank you for your comment. Following to the advice of the reviewer, added content related to setting the number of n in the protocol's pre-explained paragraph as follows:

“In order to minimize animal suffering and reduce the number of animals used in accordance with the Three Rs (Replacement, Reduction, and Refinement) guidelines, this study was designed with the minimum number of animals for significant data could be collected, established through preliminary experiment.” (Line 73~77)

3. You cannot state the animal shows symptoms of pain by observation of the wound (Fig S1).

Response: Thank you for your comment. Following the reviewer's advice, we removed the word "pain" to refer only to signs of inflammation.

Supplementary Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary Figure 1