

# Repeated Short-term (2h×14d) Emotional Stress Induces Lasting Depression-like Behavior in Mice

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Chronic behavioral stress is a risk factor for depression. To understand chronic stress effects and the mechanism underlying stress-induced emotional changes, various animal models have been developed. We recently reported that mice treated with restraints for 2 h daily for 14 consecutive days (2h-14d or 2h×14d) show lasting depression-like behavior. Restraint provokes emotional stress in the body, but the nature of stress induced by restraints is presumably more complex than emotional stress. So a question remains unsolved whether a similar procedure with “emotional” stress is sufficient to cause depression-like behavior. To address this, we examined whether “emotional” constraints in mice treated for 2h×14d by enforcing them to individually stand on a small stepping platform placed in a water bucket with a quarter full of water, and the stress evoked by this procedure was termed “water-bucket stress”. The water-bucket stress activated the hypothalamus-pituitary-adrenal gland (HPA) system in a manner similar to restraint as evidenced by elevation of serum glucocorticoids. After the 2h×14d water-bucket stress, mice showed behavioral changes that were attributed to depression-like behavior, which was stably detected >3 weeks after last water-bucket stress endorsement. Administration of the anti-depressant, imipramine, for 20 days from time after the last emotional constraint completely reversed the stress-induced depression-like behavior. These results suggest that emotional stress evoked for 2h×14d in mice stably induces depression-like behavior in mice, as does the 2h×14d restraint.

**Key words:** emotional stress, anxiety, depression, behavior

## INTRODUCTION

Depression is a prevailing form of psychiatric illness worldwide, but the underlying mechanism is not clearly elaborated. Recent progresses in the establishment of depression animal models give us a hope to unravel the molecular mechanism of depression and to develop advanced anti-depression strategies [1, 2]. Mice or rats

exposed to a variety of stressors over a prolonged period display behavioral changes that parallel depression-like symptoms, such as decreased social interaction, decreased intake of sucrose, increased immobility time in helpless conditions such as in the forced swim test and tail suspension test [3-5]. These stress-induced behavioral changes are generally reversed by chronic, but not acute, treatment with antidepressant drugs, such as imipramine or fluoxetine [4, 6], supporting the usefulness of stress-induced animal models in depression studies.

Regarding animal models, various stressors are delivered repeatedly or continuously in mice or rats for a certain period of time to recapitulate depression pathophysiology in human. Among

Received November 14, 2011, Accepted December 14, 2011

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rodent models induced by “continuous” behavioral constraints are the chronic mild stress (CMS) model [3], chronic social defeat stress [4] and prolonged isolation stress [7], while among animal models induced by “repeated” constraints are animal models with repeated restraints [8-10]. Each of these models, including others, may have their own features. However, any specific model does not have all advantages over others. For examples, the social defeat stress model introduced by Berton et al. [4] have subject animals experience an offensive aggression physically for 10 min and then emotionally and contextually through olfactory, auditory and visual stimuli by placing mice in a close proximity for the rest of the day and this procedure was repeated for consecutive 10 days. The social defeat stress model produced by this experimental procedure has a feature to provide highly robust depression-like behavioral phenotypes and molecular changes [4, 11, 12], whereas this model requires a stable provision of proper offensive animals, for example aged CD1 mice [4, 11, 13]. Given the criticism that the 10-d continuous stress exposure in the chronic social defeat stress model might be too severe, “repeated” social defeat stress paradigms have been used [14-16]. Depression-like behavioral phenotypes can be also induced in animal models treated with repeated stress, such as with 2h×10d [17], 2h×14d restraint [10], or 6h×21d restraint [18, 19]. Repeated restraints have a superior feasibility in handling experimental procedures compared to other models, although the concept of effective doses of stress threshold and optimized stress strength have not been established.

Among many questions remained to be solved, it is a challenging question to know whether depression-related behavioral changes can be stably produced in animals after what extents of “emotional” stressor are repeatedly treated. Therefore, in the present study, we examined whether lasting depression-related behavioral changes is produced by exposing mice to emotional stressor repeatedly for 2h×14d.

## MATERIALS AND METHODS

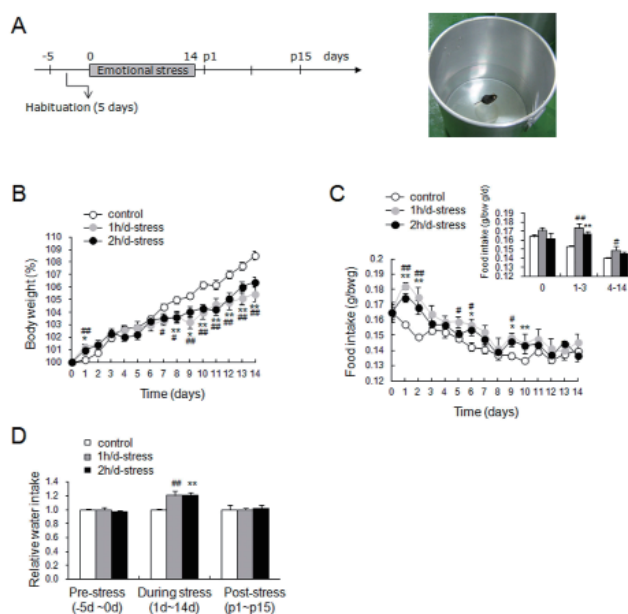
### Animals

Male C57BL/6 mice at 7 weeks of age were purchased from Daehan BioLink, Inc. (Eumsung, Chungbuk, Korea). They were housed in pair in clear plastic cages at a temperature (22~23°C)- and humidity (50~60%)-controlled environment with a 12 hour-light/dark cycle (light on at 7 a.m.) and were allowed to lab chow and water freely. They were adapted to the new environment for 1 week before use in experiments.

### Treatment of water-bucket stress

To deliver water-bucket stress, 8-week-old mice weighting

~22~23 g at 8 weeks of age were placed on a small stepping round platform (3.5 cm diameter) in a bucket (42 cm diameter x 55 cm height) with a quarter full of water. The depth of water was 17.5 cm, and the platform was elevated 1 cm above the water surface (Fig. 1A). The water-bucket stressor was delivered to animals for 1~3 h daily from 10 A.M. for 14 days on a scheduled procedure. Control mice were placed in pair in their original cages undisturbed at home environment. After each session of water-bucket stress, animals were returned to their home environment by housing them in pair in normal plastic cages with free access to food and water. Imipramine was intraperitoneally injected each day at indicated schedule. Animals were handled in accordance



**Fig. 1.** Body weight gain, food-intake, and water intake of mice treated with repeated water-bucket stress. (A) Experimental schedule of stress treatment. Mice were treated with water-bucket stress for 1~2 h daily for 14 consecutive days. A mouse enforced to stand on a confined small island in a water-pool (so named water-bucket stress) is presented (right panel). (B) Body weight changes of mice treated with water-bucket stress for 1 h or 2 h daily (1 h/d-stress and 2 h/d-stress, respectively) and their naïve control mice (control) were plotted over time. Note a reverse-S shaped pattern of body weight changes over time. (C) Food intake changes of mice exposed to water-bucket stress. After each stress treatment, mice were allowed to access to food and water freely in home cages. Inset: average daily food-intake before stress (0), for the first 3 days (days 1~3) and for the next 11 days (days 4~14) with water-bucket stress. (D) Total water intake of mice exposed to water-bucket stress and their control. Total water intake before stress (days -5~0), during stress (days 1~14) and after stress (post-stress days 1~15). The data are presented as means±S.E.M. (n=22~32). \* and \*\* denote difference between the control and corresponding data points of the 2-h stress group, and <sup>#</sup> and <sup>##</sup> denote difference between the control and corresponding data points of the 1-h stress group, at p<0.05 and p<0.01, respectively.

with The Guideline of Animal Care at Ewha Womans University School of Medicine.

#### **Corticosterone measurement**

Blood was collected from the heart of sacrificed mice at indicated time points in all cases, and centrifuged at 1,500 g for 15 min to obtain serum. Plasma corticosterone levels were assessed using a competitive enzyme immunoassay kit (R&D Systems, Minneapolis, MN, USA) as recently described [20].

#### **Behavioral assessments**

Behavioral assessments were carried out using a computerized video-tracking system, SMART (Panlab S.I., Barcelona, Spain) as previously described [10]. The mice were all placed in the same environment 30 min prior to the behavioral tests. Behavioral analyses were performed by following the sequences indicated.

**The elevated plus maze (EPM) test:** The EPM apparatus consisted of four arms (30×7 cm each) made of white Foamex, which was elevated 50 cm above the floor and placed at right angles to each other. Two of the arms had 20 cm high walls (enclosed arms), while the other two had no walls (open arms). For the EPM test, each mouse was initially placed at the center of the platform and left to explore the arms for 5 min. The number of entries into the open and enclosed arms and the time spent in each arm were recorded. Entry into each arm was scored as an event if the animal placed all paws into the corresponding arm.

**The open field test (OFT):** Locomotor activity was measured in the open field made of a white Foamex chamber (45×45×40 cm). Each mouse was placed individually at the center of the open field, and the locomotion was recorded for the indicated period. The horizontal locomotor activity was judged by the distance the animal moved.

**The tail suspension test (TST):** Mice were individually suspended 50 cm above the floor by the tail using an adhesive tape (approximately 1 cm from the tip of the tail) for 6 min. Immobility time during the last 5 min was recorded.

**The forced swim test (FST):** Mice were placed in a transparent Plexiglas cylinder (height: 27 cm, diameter: 15 cm) containing water at a temperature of 22~23°C and a depth of 15 cm so that they could not escape and could not touch the bottom. Mice were placed for 6 min in the water-filled cylinder and immobility time during the last 5 min was recorded. Immobility time was the total duration of animals remained floating with all limbs motionless.

#### **Statistical analysis**

Two-sample comparisons were carried out using the Student *t*-test, and multiple comparisons were made using one-way

ANOVA followed by the Newman-Keuls multiple range test. All data were presented as the means±S.E.M. and statistical difference was accepted at the 5% level unless otherwise indicated.

## **RESULTS**

### ***A new depression model induced by emotional stress***

Subject mice were individually enforced to stand on a small stepping platform (3.5 cm in diameter) placed in the middle of a bucket with a quarter full of water (Fig. 1A) and thereby to confront with potential drowning stress and isolation stress for hour(s). The emotional stress evoked by this procedure was termed “water-bucket” (or “small-island”) stress.

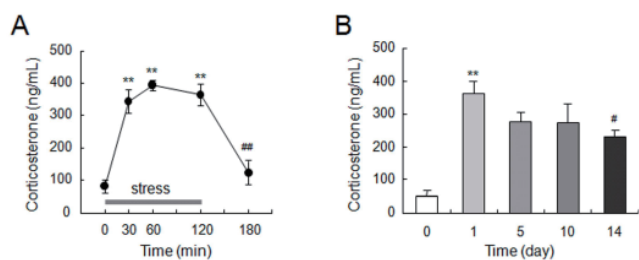
Mice (C57BL/6, 7-weeks old, male) treated with water-bucket stress for 1 or 2 h daily tended to weigh slightly more than the control for the first 1~3 days. Thereafter they gradually weighed less than the control mice until day 14. Thus, mice with water-bucket stress showed body weight changes in a reverse S-shape pattern over time (Fig. 1B), which is sharply contrast to that induced by a restraint [10]. Mice treated with 1 or 2 h of water-bucket stress ate more food than the control during the initial 1~3 days, and this tendency, though weaken thereafter, was continued to the end of the recording (Fig. 1C). Mice treated with 1 or 2 h of water-bucket stress took more water than the control during the stress period, but their water intake returned to the control level after stress (Fig. 1D).

### ***Corticosterone levels***

The water-bucket stress increased plasma corticosterone levels with ~400 ng/ml of the peak value reached ~60 min after the start of stressor exposure (Fig. 2A), indicating that the water-bucket stress potently activates the HPA axis, but its activation level is slightly weaker than that induced by the 2-h restraint [10]. After the cessation of water-bucket stress, enhanced corticosterone levels slowly returned downwards to the baseline (Fig. 2A). When this daily 2-h water-bucket stress was reapplied for next days, plasma corticosterone levels at the end of each stress session were gradually declined (Fig. 2B).

### ***Repeated (2h×14d) emotional stress induced anxiety-and depression-like behavior***

Next, we examined whether repeated water-bucket stress induces behavioral changes. In the elevated plus maze (EPM) test, mice treated with water-bucket stress for 2h×14d, but not for 1h×14d, showed lesser percentages of entries and time spent in the open arm than the control mice (Fig. 3A~C). In the open field test, however, the locomotor activities of mice in all stressed groups



**Fig. 2.** Changes in plasma corticosterone levels during stress responses. (A) Time course of plasma corticosterone levels at the end of 30-, 60-, 120-min water-bucket stress treatment, or 60 min after the 2 h water-bucket stress treatment (at post-60-min). (B) Plasma corticosterone levels at 1, 5, 10, and 14 days were measured at the end of 2 h water-bucket stress treatment. The data are presented as means±S.E.M. (n=3~12). \*\* denote difference compared with the control at p<0.01, while \* and \*\* denote difference between the 2h-stress group and indicated group, at p<0.05 and p<0.01, respectively.

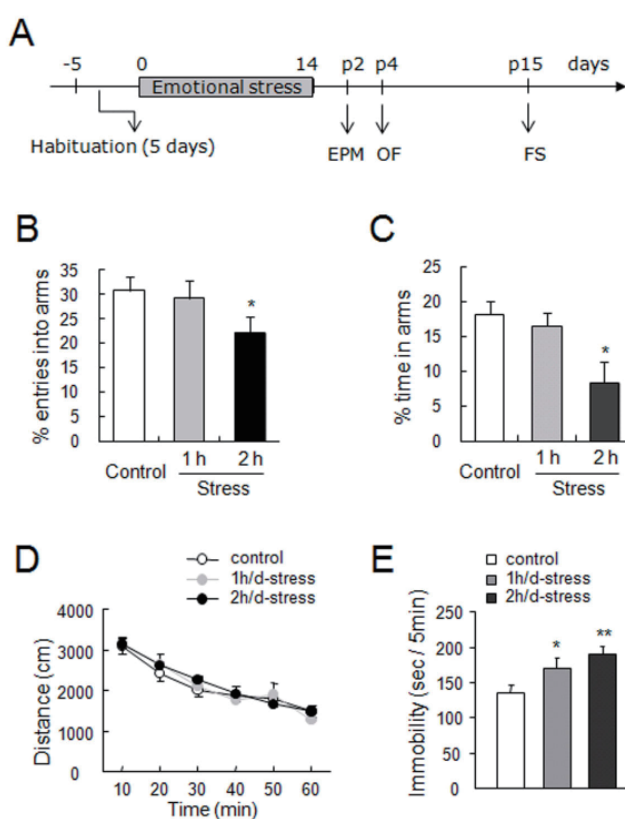
were comparable to the non-stressed control mice (Fig. 3D). In the forced swim test (FST) which was performed 2 weeks after the last stress session (Fig. 3A), mice treated with water-bucket stress for 2h×14d or 1h×14d showed a significant increase in immobility time (Fig. 3E). We observed that depression-like behavior in the FST was detected a month after the last stressor exposure (data not shown).

**The depression-like behavior induced by the 2h×14d emotional stress lasted for more than 3 weeks**

Next, we examined whether depression-like behavior induced by 2h×14d water-bucket stress was robustly persisted for 3 weeks and whether treatment of stress-mice with the anti-depressant, imipramine, for 3 weeks, gave anti-depression effects. The forced swim tests performed on post-stress day 21 (3 weeks after the last stressor exposure) revealed that mice with water-bucket stress showed enhanced immobility time in the TST (Fig. 4A, B). Similarly, the FST performed on post-stress day 22 showed that the 2h×14d stress mice displayed enhanced immobility time (Fig. 4C). In contrast, administration of imipramine in the 2h×14d stress-mice for 21~22 days from post-stress day 1 reversed depression-like phenotype in the TST and FST (Fig. 4A~C). These results suggest that 2h×14d water-bucket stress produces long-lasting changes in depression-related behavior.

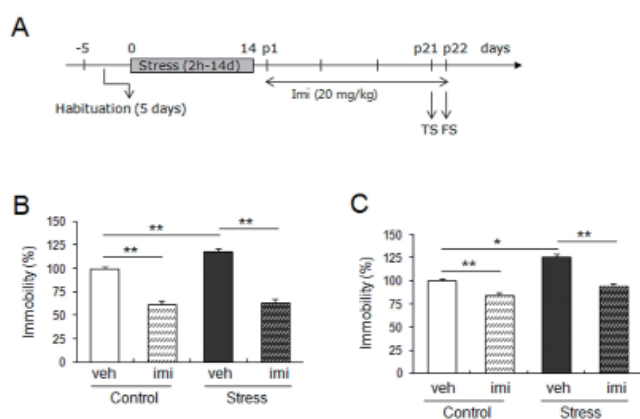
**DISCUSSION**

The present study demonstrates that emotional stress evoked by placing mice individually to stand on confined small island in a bucket with a quarter full of water (so named water-bucket stress) for 2 h daily for 14 consecutive days (2h×14d) effectively produced



**Fig. 3.** Emotional stress treated for 2 h daily for consecutive 14 days stably produced anxiety- and depression-like behavior. (A) Experimental schedule of stress treatment and behavioral tests. Behavioral analyses were performed in the sequences of the elevated plus maze (EPM) test on post-stress day 2; p2), the open field (OF) test on post-stress day 4 (p4), and the forced swim (FS) test on post-stress day 15 (p15). (B, C) Assessment of anxiety state by the EPM test. The percentages of entry numbers (B) and time (C) spent in the open arm are indicated. The 2h×7d water-bucket stress treatment did not change the emotionality on post-stress day 2 in the EPM test (data not shown). (D) The open field test. The data were plotted as distance traveled for each time block of 10 min. (E) The forced swim test. Cumulative time of immobility recorded for 5 min in the forced swim test. The data are presented as means±S.E.M. (n=7~22). \* and \*\* denote difference between the control and indicated data, at p<0.05 and p<0.01, respectively. Control, naïve mice; 1 h- and 2 h-stress, mice exposed to daily, respectively, 1 h or 2 h water-bucket stress.

a lasting change in depression-related behavior. This behavioral alteration was rescued by treatment with imipramine for 14~21 days of post-stress. We recently reported that restraint stress treated for 2 h daily for 14 days stably produced lasting changes in depression-like behavior [10]. Similar to the result of the previous study, the 2h×14d water-bucket stress activated the HPA axis and evoked anxiety- and depression-like behavioral responses. Though this 2h×14d water-bucket stress model should be strengthened by further characterization in the future, this model has a few attractive features as described below.



**Fig. 4.** Depression-like behavior induced by the 2h×14d water-bucket stress was reversed by post-treatment with imipramine. (A) Experimental schedule of stress treatment, anti-depressant treatment, and behavioral tests. Imipramine was treated at the dose of 20 mg/kg/day (i.p.) from the post-stress day 1 (from the next day after the completion of 2h-14d water-bucket stress) for 21–22 days. (B, C) Imipramine treatment reduced the immobility in the tail suspension test (TS: B) and the forced swim test (FS; C), which were performed on post-stress-day 21 and 22, respectively. The data are presented as means±S.E.M. (n=16–28). \* and \*\* denote difference between the indicated groups, at  $p<0.05$  and  $p<0.01$ , respectively.

First, the 2h×14d water-bucket depression model is based on very emotional stress. Among depression models developed on the basis of emotional stress or emotional stress-related are prey-predator conflict stress model [21–23], territorial conflict stress model [24–26], isolation stress [7], conditioned emotional stress (CES) model [27, 28], chronic mild stress (CMS) model [3, 29], social defeat stress models [4, 30–32], and restraint stress model [8, 9]. To induce depression-like behavioral changes, intended stressor(s) in these models was delivered to subject animals repeatedly or chronically, from days to weeks to months depending models. Because applied stressors and stressor-treatment procedures in these models have their own unique features, the outcome of the behavioral changes and molecular and biochemical changes in the brain might have some distinct features. In particular, because sources of stressors used in these models are varied, the neural system responding to various perceptual modalities might be complexly impinged. In the water-bucket stress model, stressor modality is relatively simple and very emotional. In addition, a stable induction of depression-like behavior was provoked by the defined procedure, and its tied behavioral correction by treatment with imipramine supports for the usefulness of the 2h×14d stress paradigm to generate a depression model in mice. Future studies to reveal associated neural circuit for evoking emotional stress will be achieved using this model.

Second, the 2h×14d water-bucket stress procedure is composed

of relatively mild stress, but this 2h×14d stress stably produces relatively long-lasting changes in depression-related behavior. The water-bucket increased plasma corticosterone level rapidly (Fig. 2), but the peak level was slightly lower than that induced by restraint and time-dependent decline upon repeated stress was also milder than that induced by restraint [10], suggesting that water-bucket stress activates HPA axis slightly milder than restraint stress does. The time-dependent decline in plasma corticosterone levels implies that the feedback-loop actively worked out in the body, but its requirement in the 2h×14d water-bucket stress model was less strongly active. Concerning the chronic social defeat stress model introduced by Berton et al. [4], it was produced by a continuous 10-days stress exposure, which consisted of daily 10-min defeat stress followed by enforcing subject mice to closely stay with an aggressor for the remainder of the day. Regarding isolation stress model, mice exposed to isolation stress for 3 months show anxiety- and anhedonia-like symptoms [7]. Isolation stress is a chronic mild stress, but the experimental procedure takes too long or require big patient to repeat in a regular laboratory. While the chronic mild stress (so called CMS) is given by multiple combinations of various unpredictable stressors within the CMS schedule [3, 29]. For examples, mice or rats are exposed to 85 dB noise for 3 h, water deprivation for 20 h, 45 degree cage tilting, wet cage-floor, repeated cold stress (4°C), empty cage, cage shaking, light-on for all day, and/or low intensity stroboscopic illumination (300 flashes/min) for 9 h, are combinatorially and continuously administered for a period of 4–8 weeks. These stressors as a whole appear not to be milder compared to isolation stress and water-bucket stress, although the total strength of stress among different models might be difficult to compare precisely, particularly when stress expose is prolonged.

Third, the 2h×14d water-bucket stress paradigm produces depression-like behavior which is long-lasting. Clinically used antidepressants usually need to be treated for 1–3 weeks to obtain antidepressant effects [33, 34]. Recent studies suggest that depression is produced by epigenetic, biochemical and structural alterations in the brain [4, 11, 12, 35]. Because the 2h×14d water-bucket stress model is developed to have a window of more than 3 weeks from the day after the last stress session until behavioral tests, this model permits an opportunity to evaluate the efficacy of potential anti-depressants and their underlying mechanisms. Moreover, the 2h×14d water-bucket stress paradigm produces long-lasting behavioral changes, which will help us unravel the mechanisms underlying depression and develop novel strategies for anti-depression.

In conclusion, the present study demonstrated that the 2h×14d water-bucket stress, though mild and emotional, causes a lasting

change of depression-like behavior, an effects that are produced by the 2h×14d restraint. Moreover, the present study demonstrated that repeated water-bucket stress model can be used to test of the efficacy of potential anti-depression drugs.

#### ACKNOWLEDGEMENT

This research was supported by a grant (2010K000814) from Brain Research Center, The 21st Century Frontier Research Program of the Ministry of Education, Science and Technology, Republic of Korea.

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