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Research article

# Development and optimization of psychological stress model in mice using 2 level full factorial design

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ABSTRACT

*Introduction:* Psychological stress has long been a silent killer, impairing normal physiological functions and leading to a variety of diseased conditions. However, the existing animal models for studying psychological stress have been marred by their inherent limitations warranting further research in their development and optimization.

*Methods:* In this study $2^5$ full factorial design was utilized for the development and optimization of psychological stress model in mice by applying different stressors *viz.*, slanted cage($X_1$), restraint($X_2$), no bedding($X_3$), dirty bedding($X_4$) and isolation($X_5$) at two time duration levels of 30 and 60 min. The development of behavioral changes like depression, anxiety and anhedonia was taken as criteria for development of stress. These responses were analyzed using Design Expert 7.1.6. (Stat-Ease, Inc., USA). The maximum effective responses obtained were taken as a criterion for optimization. The optimized model was applied to measure the change in serum cortisol level to confirm the stress development.

*Results:* The statistical data showed that a quadratic model was fitted to the data obtained. All the factors were found to have a significant role in the development of stress among which restraint, slanted cage and dirty bedding were found to be more causal ($p < 0.05$). Serum cortisol level was increased significantly in the stressed mice of optimized model ($p < 0.05$).

*Discussion:* Utilizing the magnitude of responses from the quadratic equations, it can be concluded that slanted cage, restraint and dirty bedding stressors should be applied for longer duration than other stressors for psychological stress development in mice. The study could lay a strong platform for the use of quality by design approach in the development of robust, efficient and resourceful animal models.



## 1. Introduction

Psychological stress is a prevailing facet of daily life and is considered as a risk factor for the development of diseases like, anxiety, depression, cardiovascular disease, and immune suppression (McEwen, 2002). Clinical studies showed that stressful life events produce a negative impact on physiology and behavior of human beings. In order to explore the impact and mechanism of psychological stress, some chronic unpredictable/mild stress (CUS/CMS) models have been developed and used to induce a number of endocrinological, neurological and behavioral changes in rodents (Willner et al., 1992; Di Chiara et al., 1999; Kioukia-Fougia et al., 2002; Bekris et al., 2005; Gu et al., 2009). However, each model has inherent limitations and none of them can reproduce all of the effects. Moreover, these animal models employed psychological (*e.g.*, isolation/overcrowding), physical (*e.g.*, cold/heat), and physiological (*e.g.*, insulin/lipopolysaccharide) stressors. These models suffered a major drawback of not fully reproducing stress related physiological and biochemical changes simultaneously. Among these models, some reproduced physical stress and associated neuroendocrine changes (Kvetnansky, & Mikulai, 1970), whereas others were better at producing psychological stress and associated behavioral changes (Marcelo et al., 2007). Additionally, they suffered a major drawback of development of resistance on chronic application, showing diminished response. Thus, there is a need to develop a stress model paradigm that is robust, reproducible, avoids adaptation and mimics the natural progression of psychological stress.

The outcome of an animal experiment is influenced by a number of factors. Factorial design (FD) allows the determination of the effect of these factors and their interactions with minimum number of experiments (Box et al., 1978). It is also useful to determine which factors are relatively unimportant in influencing response so that less attention is given to controlling them. The factors that can be considered include direct animal-related characteristics (*e.g.*, sex, strain, age, and dietary and health status), environmental factors (*e.g.*, cage and group\size, bedding material, and environmental complexity) and protocol-specific

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factors (*e.g.*, time of start of experiment; methods and timing of observations dose level; route, and method of administration of test compounds).

FD ($2^k$ series) has been widely used in the manufacturing industry as a means of maximizing output for a given input of resources (Cox, 1958; Montgomery, 1997). Though FD has not been utilized in biological systems extensively, but there are few reports in which FD was used to optimize biological parameters. FD methods were used to optimize enzyme linked immunosorbent assay tests (Reiken et al., 1994), conditions for freezing rat liver slices (Maas et al., 2000), DNA microarray experiments (Wildsmith et al., 2001) and to assess the effects of medium composition, incubation conditions, and associated microflora on the production of type G Clostridium botulinum toxin (Calleri de et al., 1992) *in vitro*. A few *in vivo* studies have also been reported *viz.*, effect of genotype, diet, and exercise in the accumulation of body fat in rats (Metzger et al., 2000); the effects of strain and dose level of chloramphenicol on mouse haematology (Festing et al., 2001); the effects of carcinogenic mixtures on the development of lung tumors in mice (Nesnow et al., 1998) and the impact of housing condition in animal model of depression and anxiety (S'aenz et al., 1996). However, in some of these cases, FDs have been used to gain an understanding of the factors influencing the observed response, rather than to optimize future experiments.

In the present study, psychological stress model was developed and optimized by utilizing a $2^5$ full factorial design to evaluate the combined effect of selective independent variables on various behavioral changes that are considered as behavioral stress markers. The study demonstrates the use of factorial design to proceed systematically in the study of multiple factors by avoiding the usage of large amount of animals.

## 2. Materials and methods

### 2.1. Animals and housing

Female *Swiss albino* mice of 6–8 weeks of age, weighing 25–30 g were obtained from the animal house of B. V. Patel PERD Centre, Ahmedabad. Animal housing and handling were performed in accordance with Good Laboratory Practice (GLP) mentioned in CPCSEA guidelines. Animal house is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India, vide registration no. 1661/PO/Re/S/12/CPCSEA, dated 26/10/2015. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee prior to initiation of the experiment (Registration No. PERD/IAEC/2015/020). The mice were housed in polypropylene cages (five animals per cage) which were then kept in isolation and were allowed to acclimatize for two weeks before experiment. A 10% air exhaust conditioning unit was maintained along with a relative humidity of 60 ± 5% and a temperature of 25 ± 3 °C in the animal house facility. A 12:12 h light:dark cycle was also regulated for the experimental animals.

### 2.2. Experimental protocol

In this study a $2^5$ full factorial design was used to optimize psychological stress model in mice. The factors *viz.*, slanted cage($X_1$), restraint($X_2$), no bedding($X_3$), dirty bedding($X_4$) and isolation($X_5$) were selected as independent variables. Each factor was applied for two time duration levels *viz.*, 30 min and 60 min. The actual values and coded values are given in Table 1. Thirty two different psychological stress models (T1 to T32) were prepared according to the design as shown in Table 1. Previous studies showed that adolescent' females are more prone to felt stress than males (Bird and Harris, 1990), therefore in this study we utilized female mice to develop a psychological stress model. The percentage sucrose preference, percentage exploration in open arm and period of immobility were taken as response parameters.

### 2.3. Psychological stress model

Psychological stress model consisted of exposure (started at 10:00 h) to each stressor as shown in experimental design (Table 1) for one day. The types of stressors were selected to challenge the animal psychologically (Barnum et al., 2012). Each stressor was applied at two time duration level *viz.*, 30 min and 60 min. For each test session, n = 3 mice were used. After the stress sessions, behavioral parameters were evaluated in mice.

### 2.4. Assessment of behavioral changes due to psychological stress

Each behavioral test was carried out by the same person on the same time to avoid variability in observations. All mice were tested immediately after the test sessions.

#### 2.4.1. Sucrose preference test

Sucrose preference test is often used as an indicator of anhedonia. A two-bottle (one with 2% w/v sucrose and other with water) choice paradigm was used to test the stressed mice for their relative preference for sucrose over water (Barnum et al., 2012). No previous food or water deprivation was done before the test. The preference for sucrose was calculated in percentage by using the formula given below. A sucrose preference below 65% was taken as the criterion for development of anhedonia.

$$\text{Sucrose preference} = (\text{Volume of sucrose solution consumed}/\text{Total volume of liquid intake}) \times 100$$

#### 2.4.2. Forced swim test

The test procedure was carried out according to the method described by Porsolt, et al. (1977) with some modifications. Mice were introduced to a glass beaker (height 25 cm, diameter 10 cm) filled with warm water (25 °C, height 10 cm). Animals were allowed to swim for 6 min and the period of immobility, defined as the absence of directed movements of animals' head and body were estimated during the last 4 min of the test. Water in the beaker was changed with every mouse. The total immobility time was defined as the total amount of time in which mice remained immobile or makes only small limb movements necessary for floating. Immobility time was recorded with a stopwatch and behavior was analyzed by a trained observer.

#### 2.4.3. Elevated plus maze test

The development of anxiety was assessed as a response to novel situation that produces unconditioned avoidance responses towards the open arms. The elevated plus maze consists of two open and two closed arms (16 × 5 × 12 cm), 25 cm above the floor. Each mouse was placed in the central square (5 × 5 cm) facing an open arm, and allowed to explore the maze for 5 min, during which the following measures were taken: number of entries and time spent in the open arms, number of entries and time spent in the closed arms and total number of arm entries (Kulkarni, 1999). An entry was counted when animal was on an arm with all four paws. The maze was cleaned with 5% v/v ethanol solution and water after each trial. The percentage exploration onto open arms was taken as measures of anxiety.

### 2.5. Analysis of responses by design expert

Design Expert 7.1.6. (Stat-Ease, Inc., USA) was used for the design and analysis of effect of each variable on the designated response. Pareto charts were made for the analysis of each response coefficient for its statistical significance. Quantitative and qualitative contribution of each variable on each of the response was analyzed. The significant response polynomial equations generated by Design Expert were used to validate