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An Evaluation of the Effects of Two Chronic Immobilization Stress Protocols on Depression/Anxiety-Related Behavior in Male Rats

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ABSTRACT

Objective: The effect of acute and chronic stress models on depression and/or anxiety-like behavior in rodents has been widely studied, but with contradictory results. This may be due to differences in the sex and age of the animals studied or inherent differences in the stress models used. Therefore, this study aimed to evaluate the effects of two immobilization stress protocols on depression/anxiety-like behaviors in adult male rats.

Materials and Methods: Adult Wistar rats were randomly divided into three groups (n=10) comprising: control, immobilization stress-1 (45 minutes daily for a period of ten days), and immobilization stress-2 (45 minutes twice a day for a period of ten days). Stress-related behavior was evaluated by means of the open field and forced swim tests. In addition, change in body weight, fasting blood glucose, and serum corticosterone were measured.

Results: In the open field test, the percentage of time spent in the central area and mean velocity were significantly lower in the immobilization stress-1 and immobilization stress-2 groups as compared to the control group ($p < 0.05$ and $p < 0.01$, respectively). Movement ratios were lower in both immobilization stress groups than in the control group ($p < 0.001$ and $p < 0.01$, respectively). In the forced swim test, the duration of swimming, climbing and immobility behavior in both immobilization stress protocols did not differ from the control group. Serum corticosterone levels were higher in the immobilization stress-1 and immobilization stress-2 groups than in the control group ($p <0.05$), but no overt differences were determined in the percentage change in body weight or the fasting blood glucose level between the stress protocol groups and the control group ($p > 0.05$).

Conclusion: We may conclude that immobilization stress-1 and immobilization stress-2 protocols do not cause depression-like behavior in adult male rats. However, anxiety-like behaviors predominated in both stress protocol groups.

Keywords: immobilization stress, depression, anxiety, open field, forced swimming test

İKİ KRONİK İMMOBİLİZASYON STRES PROTOKOLÜNÜN ERKEK SIÇANLARDA DEPRESYON/ANKSİYETE BENZERİ DAVRANIŞLAR ÜZERİNE ETKİLERİNİN DEĞERLENDİRİLMESİ

ÖZET

Amaç: Kemirgenlerde akut ve kronik stres modellerinin depresyon ve/veya anksiyete benzeri davranış gelişimine etkilerine yönelik çalışmalar oldukça fazla olmasına karşın çelişkili sonuçlar da söz konusudur. Bu durum hayvanların cinsiyet ve yaşlarına bağlı olabileceği gibi kullanılan stres modellerinde farklılıklarla da ilişkili olabilir. Bu nedenle, söz konusu çalışmamızda erişkin erkek sıçanlarda iki immobilizasyon stres protokolünün depresyon/anksiyete benzeri davranışlara etkisinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Erişkin Wistar ırkı sıçanlar kontrol, immobilizasyon stresi-1 (on gün boyunca günlük 45 dakika) ve immobilizasyon stresi-2 (on gün boyunca günde iki kez 45 dakika) olmak üzere rastgele üç gruba (n= 10) ayrıldı. Stresle ilgili davranışlar açık alan testi ve zorunlu yüzme testi ile değerlendirildi. Ayrıca, vücut ağırlığı değişimi, açlık kan glikoz seviyesi ve serum kortikosteron düzeyi de ölçüldü.

Bulgular: Açık alan testinde, immobilizasyon stresi-1 ve immobilizasyon stresi-2 gruplarında merkez alanda harcanan zaman yüzdesi ve ortalama hız kontrol grubuna kıyasla önemli düzeyde düşüktü (sırasıyla $p <0.05$ ve $p <0.01$). İmmobilizasyon stres gruplarında hareket oranlarının kontrol grubuna göre daha düşük olduğu belirlendi (sırasıyla $p <0.001$ ve $p <0.01$). Zorunlu yüzme testinde, yüzme, tırmanma ve immobilize davranış süreleri her iki immobilizasyon stres protokolünde de kontrol grubundan farklı değildi. İmmobilizasyon stres protokolü 1 ve 2'de serum kortikosteron düzeyi kontrol grubundan daha yüksekti ($p <0.05$), fakat vücut ağırlığı değişimi ve açlık kan glikoz düzeyinde istatistiksel bakımdan farklılık söz konusu değildi ($p> 0.05$).

Sonuç: Yetişkin erkek sıçanlarda immobilizasyon stresi-1 ve immobilizasyon stresi-2'nin depresyon benzeri davranış profiline neden olmadığını, fakat her iki stres protokolünde de anksiyete benzeri davranış profilinin ön planda olduğu söylenebilir.

Anahtar sözcükler: İmmobilizasyon stresi, depresyon, anksiyete, açık alan testi, zorunlu yüzme testi, erkek sıçan

Anxiety and depression are widespread within the general population (1). These disorders develop through a complex interaction between stressful living conditions and genetic predisposition (2,3). These factors alter psychophysiological function, meaning emotions, behaviors and neuroendocrine activity, in both humans and animals (4,5).

Animal models are an important resource for trying to understand the pathophysiological mechanism of stress-induced neuropsychiatric disorders and for developing novel therapeutic agents for the management of stress (6). Small rodents, especially mice and rats, are a key option for use as research models where a whole body system is required, since experiments on such animals are economical in terms of research costs and housing, because they can be easily bred and have a small body structure. Although they are biologically similar to humans in terms of the functional systems implicated in the stress response, there are some fundamental differences between these animals and humans,which may substantially affect the interpretation of results obtained from these studies (7). For these reasons, it is very important to evaluate which model is most appropriate for neuropsychiatric research.

There are a number of models for inducing stress in small rodents (8). Immobilization or restraint stress is one of the most popular experimental models used to evaluate the stress-related physiological responses and the anti-stress activity of pharmacological agents in animals (9,10), in particular because restraint stress is both effortless and painless (10). However, there are many variations between how these models are implemented with respect to differences in the equipment and size of restrainers as well as the time interval for restraint (10,11). Although both procedures are considered similar, or indeed,equivalent, the immobilization stress model may be a more intense stressor than the restraint stress model (6,8). In the chronic immobilization stress model, many researchers have employed variable time periods ranging from 5 to 30 days to induce chronic stress of varying degrees in mice and rats (6). To the best of our knowledge, no study has so far reported on the relationship between anxiety/depression-type behavior and the immobilization stress protocol involving a plexiglass tube in male rats induced by daily 45 minute periods for ten days or twice a day 45 minute periods for ten days. Therefore, we aimed to evaluate the effects of two different chronic immobilization stress protocols on depression/anxiety-related behaviors in male rats.

Materials and methods

Animals

Thirty adult male Wistar albino rats weighing 360-390 g (KONÜDAM Experimental Medicine Application and Research Center of Necmettin Erbakan University, Konya) were used in this study. The animals were housed under a standard light/darkness schedule (12-h light/12-h dark cycle), at constant temperature (21 ± 1°C) and humidity (55±5%) with free access to pelleted food and tap water.

Ethical approval

All experimental protocols in the present study were approved by the Local Ethical Committee of Necmettin Erbakan University and the animals were treated in accordance with national and international laws and policies on the care and use of experimental animals.

Immobilization stress protocols

Rats were randomly divided into three groups (n=10 for each group): control, immobilization stress-1 (45 minutes daily for a period of ten days), and immobilization stress-2 (45 minutes twice a day for a period of ten days).

For immobilization stress, our own method, which was modified from its use in previous studies (12, 13), was used. The rats were placed in a cylindrical apparatus, which was suitable for their body volumes: 6.5 cm × 6.5 cm × 22 cm in dimensions (Figure 1A). Such cylinders were made from transparent plexiglass. There were ventilation holes in the walls of this apparatus in the parts surrounding the animal body. The same holes were also present at the front of the head section. This plastic front section was designed to prevent head movement, and was adjustable to the length of the animal body (Figure 1B). At the back the cylinder was perforated and had lockable sliders to allow the tail of the animals to remain outside the apparatus (Figure 1C). After the animals had been placed in the apparatus, the head section could be moved backwards by between 22 cm and 5 cm with the aid of a slider, adjusted according to the length of the animal (Figure 2A). Thus, the cylindrically-shaped, collapsed area ensured immobilization of the animal's limbs as well as their head movements (Figure 2B). At the same time, whilst the experimental procedure was being carried out on the stress group, the control group animals were held several times and then returned to their cages.

Open field test and forced swimming test

Anxiety and depression-like behaviors were evaluated in rodents following ten days by open field and forced

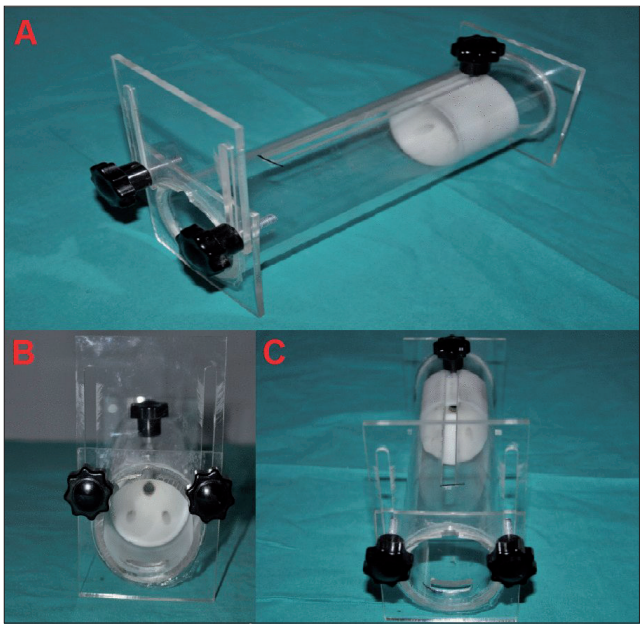

Figure 1. Transparent plexiglass cylinder apparatus for immobilization stress

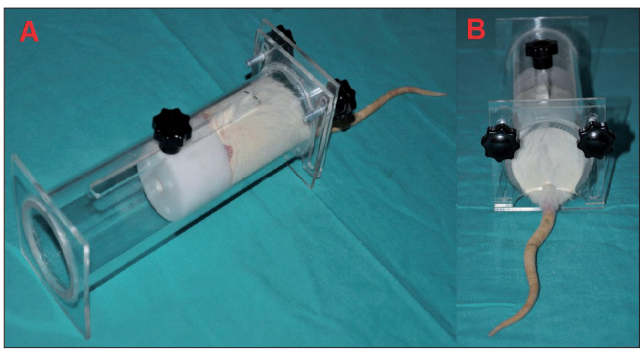

Figure 2. Immobilization stress induction

swimming tests. The open field test is generally used to determine anxiety-related behavior in rodents (14). On the 11th day, the test was performed between 9.00 and 11.00 am. The rats were placed in the center of a square-box test apparatus (80×80×30 cm, black Plexiglas) and tracked using a video tracking system (Ethovision 11, Netherlands) for a period of 300 seconds. On the software screen, the platform surface was divided into center and edge regions. Scores for time spent in the central area (s), movement ratio (%), and mean velocity (cm/s) were calculated by the Ethovision software. Rearing and grooming behaviors were manually scored by reviewing the video records produced by the software.

The forced swimming test, first described by Porsolt et al. (15), is widely used to analyze depression-like behavior in rodents. This test was performed according to our previously developed protocol (16). On the 12th day, for the pretest session, the animals were placed individually into plexiglass cylinders (49 cm height, 25 cm diameter) containing 39 cm of water (27±1°C) for 15 min. 24 h after the pretest session, the forced swimming test was performed. Scores for swimming, climbing, and immobility behavior were calculated by the Ethovision XT11 video tracking system for a period of 300 seconds.

Fasting blood glucose and serum corticosterone analyses

At the end of the anxiety and depression tests, the rats were fasted overnight although water was provided *ad libitum*. On the day they were to be sacrificed, the animals were weighed and anesthetized with a cocktail of xylazine/ketamine (8/60 mg/kg, intramuscular). Trunk blood samples were obtained by decapitation. Fasting blood glucose levels were measured using a manual glucometer (Optium Xceed, UK).

Serum corticosterone levels were analyzed according to the methods previously used, with some modifications (17). In brief, 1 µg/mL of corticosterone-BSA as stock solution was diluted with carbonate buffer (pH 9.6), and this solution then transferred (200 µl/well) into a 96-well microtitre plate (Nunc, Roskilde, Denmark). After overnight incubation at +4°C, the plate was washed with washing buffer and blocking buffer (200 µl/well) was added for 120 min at 37°C. The plate was washed, and serum samples or standards (50 µl/well) were preincubated with primary antibodies (50 µL/well) for 45 min at 37°C and then transferred into coated plates for competition with antigens on the solid phase for 30 min at 37°C. After washing, 100 µl/well biotinylated goat anti-rabbit IgG was added, and the plate was incubated for 30 min at 37°C. The plate was washed, 100 µl/well streptavidin peroxidase solution (Sigma-Aldrich, Taufkirchen, Germany) was added, and the plate was incubated for 15 min at 37°C. After washing, tetramethylbenzidine substrate (150 µl/well) was added, and the plate was incubated in the dark for 10 min. Stop solution (sulfuric acid 10%, 50 µl/well) was added, and the absorbance was measured at 450 nm using a microplate reader (Biotek, Synergy HT, USA). The dynamic range of the assays was between 10-2000 ng/ml. Inter- and intra-assay coefficients of variation were below 10%.

Statistical analysis

All results were expressed as mean ±SEM. The differences between groups were evaluated using one-way ANOVA with *post hoc* LSD test using the SPSS Software. $P < 0.05$ was considered to be statistically significant.

Results

As seen in Table 1, the percentage of time spent in the central area was significantly lower in the immobilization stress-1 and immobilization stress-2 groups as compared to the control group ($p < 0.05$ and $p < 0.01$, respectively). Movement ratios were lower in both of the immobilization stress groups than the control group ($p < 0.01$). The mean velocity of the immobilization stress-1 and immobilization stress-2 groups was significantly lower than that of the control group ($p < 0.05$ and $p < 0.01$, respectively). The scores for grooming and rearing behavior did not differ between groups.

The values reflecting the effect of two chronic immobilization stress protocols on the scores for swimming, climbing, and immobility behavior in the forced swimming test are presented in Figure 3. The duration of swimming and climbing behaviors was not affected by either of the stress protocols. Immobility behavior was statistically unchanged by the immobilization stress-1 and immobilization stress-2 protocols, although it showed a tendency to increase in the immobilization stress-1 group compared to the control group.

Serum corticosterone levels were found to be higher in rodents from both stress protocol groups, compared to the control group ($p < 0.05$, Figure 4). The percentage change in body weight and the fasting blood glucose level were not significantly altered by the immobilization stress-1 and immobilization stress-2 protocols. (Table 2). There was no statistically significant difference in any analyzed parameters between the immobilization stress-1 and immobilization stress-2 protocols.

Table 1. Effects of two chronic immobilization stress protocols on anxiety-related behaviors of rats assessed by means of the open field test

Parameters	*Control*	*Immobilization stress-1*	*Immobilization stress-2*
Time spent in central area (%)	2,1±0,4	0,9±0,4[a]	0,6±0,1[b]
Movement ratio (%)	72,2±7,9	53,6±2[b]	52,4±1,1[b]
Mean velocity (cm/s)	9,02±1,1	6,3±0,6[a]	5,8±0,5[b]
Rearing frequency	23,5±2,06	21,7±1,9	20±2,6
Grooming frequency	3,5±0,5	3±0,7	3,2±0,2

Values plotted are mean±S.E.M (n= 10 for each group).[a] $P < 0.05$, [b] $P < 0.01$, and [c] $P < 0.001$ compared to the control group.

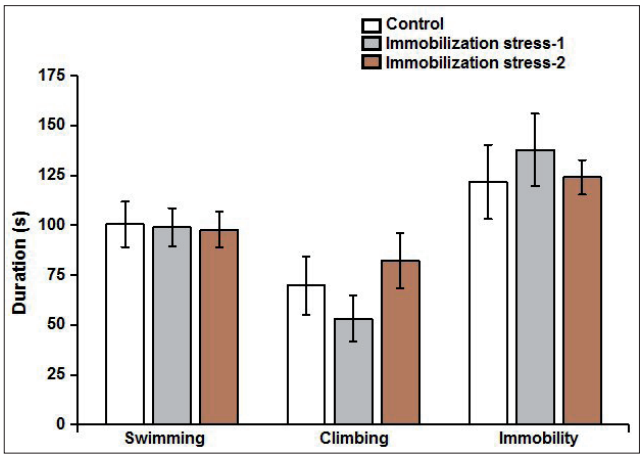

Figure 3. Effects of two chronic immobilization stress protocols on depression-like behaviors of rats assessed by using the forced swimming test. The plotted values are mean±S.E.M (n= 10 for each group).

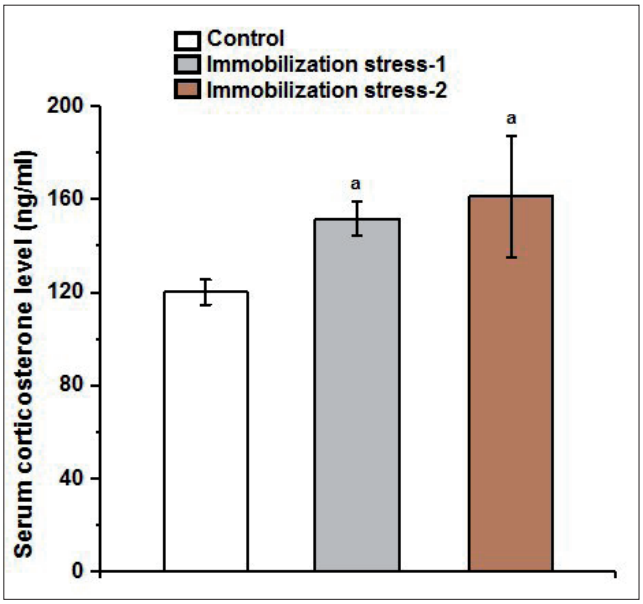

Figure 4. Effects of two chronic immobilization stress protocols on serum corticosterone level in adult male Wistar albino rats. Plotted values are mean±S.E.M (n= 10 for each group). $P < 0.05$, compared to the control group.

Table 2. Effects of two chronic immobilization stress protocols on percentage change in body weight and fasting blood glucose level in adult male Wistar albino rats

Parameters	*Control*	*Immobilization stress-1*	*Immobilization stress-2*
Change in BW (%)	3,6±0,7	4,5±0,9	3,6±0,3
Glucose level (ng/dl)	48,4±2,9	49±4,1	44,1±1,3

Values plotted are mean±S.E.M (n= 10 for each group). **BW:** Body weight

Discussion

The present study highlights the results of two immobilization stress protocols performed in an attempt to identify an effective and useful model for use in future studies of anxiety and/or depression. Our results indicate that

both immobilization stress-1 (45 minutes once daily for a period of ten days), and immobilization stress-2 (45 minutes twice a day for a period of ten days) protocols induce anxiety-like behaviors in adult male rats. In the open field test, the percentage of time spent in the central area for the immobilization stress-1 and immobilization stress-2 groups was lower than the control group. Generally, a decrease in the time spent in the central area in the open field test is accepted as indicative of high anxiety or fear (18,19). The decrease in percentage of time spent in the central area indicates that the animals prefer to be around the edge rather than in the central region, due to their anxiety. Although there was no significant difference in grooming and rearing behaviors, the movement ratio and velocity were reduced in both immobilization stress groups. In previous restraint or immobilization stress studies, it has been reported that anxiety-related behaviors increase in rats after exposure to these stress protocols (20,21). The findings from our study are consistent with these results. In the forced swimming test, duration of swimming and climbing behaviors in both immobilization stress protocols did not differ from the control group. The scores for immobility behavior were higher in the immobilization stress-1 group, but this increase was not statistically significant. In addition, change in body weight and fasting blood glucose levels did not differ between the stress protocol groups and the control group. These results can be interpreted as indicating that neither immobilization stress-1 nor immobilization stress-2 causes depression-like behavior. Thus, immobilization stress may lead to increased depression-like behavior or increased levels of biomarkers when compared to restraint stress, and it may be a useful tool for evaluating antidepressant drugs (22). However, there are many different protocols, both acute and chronic, producing different results within this stress model (6).

We conclude that our applied stress model is comparable to the immobilization stress procedure, and the results obtained fit with this method. In rodents, the immobilization and restraint stress models are based on exposure to movement restriction. The restraint model is a procedural variation of the immobilization stress model (6). These models are generally considered equivalent, except for the intensity factor, but in fact there are some technical differences between the two models (10,11). The immobilization stress protocol was developed by Kvetnansky and Mikulai (9) and this model has now become one of the most frequently used stress protocols for rats and mice (6). A typical immobilization stress protocol involves fixing the four limbs of small rodents in prone position on a plain board with adhesive tape. The heads of the animals are also fixed with a metal loop over the neck region to restrict head movements (9). Currently, immobilization stress can also be induced by fixing the limbs of the animal in an adjustable plastic bag, a transparent plexiglass cylinder or other equipment (12,23,24). Restraint stress is generally performed by keeping the animals in small wire mesh cages, a cylindrical or semi-cylindrical tube with ventilation holes for a stipulated period of time (25-27). In restraint stress, although the range of animal movement is seriously limited, the limbs are not secured and the animal remains within an enclosed area (25). Moreover, this stress model does not include limitation of head movement. Therefore, it can be assumed that immobilization stress is a more intense stressor than the restraint model (6,8). However, information within the literature pertaining to the difference in these protocols is severely limited due to the absence of technical detail for the immobilization and restraint protocols (10). There is also no strict definition of the two protocols available because they are often used interchangeably. For instance, recently, Marmonti et al. (28) have described a new immobilization model designed to mimic the situation encountered in humans in bed rest. In this model, rats were confined to a reduced space in a cage that had its volume reduced by 80% compared to the standard control cage. This setup meant locomotor movements of the animals were restricted for days or weeks at a time, but without any accompanying restriction of food or drinking water. However, we propose that the mentioned model be classifies as a restraint stress or muscle disuse model for rodents because the movement of the limbs, body and head of the animals is not prevented, only restricted.

We do not suggest that our application is an altogether new model because immobilization through the restriction of locomotion is not a novel approach in behavioral neuroscience research, particularly in the study of stress response (10,22). Our main focus is the definition of an effective immobilization method with respect to the two different time-based procedures and interpretation of the results obtained. There are, in fact, multiple different procedures, which are generally categorized under immobilization or restraint stress, with results subject to some debate, and based on the type of equipment used (Plexiglas tube, wire mesh restrainer or restraint cage), duration and frequency of applied stress (6,10). There are some similar models in existence, although they are not identicalin duration or frequency of restraint/immobilization. In a study, it was found that prenatal restraint stress, induced three times a day for 45 min for a period of 10 days, decreases

motor activity levels in adulthood (29). However, anxiety, depressive behavior, and stress or corticosterone changes were not defined in this particular study. In another study, it was reported that repeated immobilization stress in male rats (a session lasting 180 minutes, once a day for 10 days) did not affect plasma corticosterone levels, but increased corticotrophin releasing factor-immune reactivity in the median eminence, though not in other brain regions, at 24 hours time post-stress (30). By contrast, the two immobilization stress models used in our study caused anxiety-related behavior as well as serum corticosterone alterations. The serum corticosterone level increased in both immobilization-1 and immobilization-2 protocols.

Conclusion

In conclusion, we suggest that the stress models used in this study conform to immobilization stress because these techniques have the properties required for an immobilization procedure. We used two immobilization models, immobilization-1 (45 minutes daily for a period of ten days), and immobilization-2 (45 minutes twice a day for a period of ten days), both of which caused a significant increase in anxiety-like behaviors. Moreover, serum corticosterone levels were elevated in both stress groups. There was no statistical difference in the scores for anxiety-related behavior nor in corticosterone levels between the immobilization stress-1 protocol and the immobilization stress-2 protocol. This result may be related to stress adaptation. Therefore, we propose that daily 45 minute periods for ten days can be used as an effective immobilization model for stress or anxiety studies conducted using male rats.

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Conflict of interest statement

The authors report no conflicts of interest.

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