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Pharmacological Investigation of Serial Anxiety Tests in the Mouse: A Pilot Study

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AMU and MGM conceptualized and designed the study, carried out the experiment, analysed data, interpreted results and drafted the first manuscript. Authors OSB, CA and MIA did the proof reading. All authors read and approved the final manuscript.

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ABSTRACT

The use of test batteries has been suggested as a means to enhance throughput, to broaden the spectrum of anxiety parameters detectable and to minimise the numbers of experimental animals required in behavioural studies. This study was undertaken to determine the sensitivity of test batteries each of open field, zero mazes and staircase tests to anxiety behaviours in randomised mouse groups. Mice (n = 8) were exposed to these apparatuses serially in that order, thirty minutes following intraperitoneal administration of distilled water, 0.5 and 1.0 mg/kg diazepam. Each mouse was allowed to spend 5 minutes to freely explore each test apparatus. The results showed

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diazepam-treated mice exhibited significantly ($p<0.05$) reduced anxiety behaviours compared to the placebo group on most rodent anxiety parameters evaluated. The findings of this study suggest these behavioural test apparatuses, when used in serial combination, are sensitive and reliable to measure murine anxiety-related behaviours and the anxiolytic effects of standard/putative agents.

Keywords: Evaluation; open-field; elevated zero-maze; staircase; mice.

1. INTRODUCTION

Anxiety disorders are a highly prevalent class of mental disorders characterized by inappropriate, unregulated, frequent, persistent or excessive expression of anxiety or fear [1]. The high prevalence and the attendant huge socio-economic burden of these disorders viewed against the limitations of the existing anti-anxiety drugs indicate a need for discovery of additional anxiolytic agents [2,3,4,5,6].

The discovery of putative anxiolytic agents is often initiated by the use of a variety of animal anxiety tests to model human anxiety disorders [7,8]. Among several animal anxiety models, rodent anxiety tests have been shown to play an important role in screening for potential anxiolytic agents during the preclinical stages due to their robust predictive and translational validity for both human anxiety and pharmacological evaluation of the anxiolytic activity of standard and novel drugs [8,9,10,11].

To facilitate throughput in this pilot and an anticipated main study, test batteries each of open field, zero mazes, and staircase were generated and arranged in series in that order. The generation of multiple test batteries was to afford parallel testing of subjects from all experimental groups to minimize inter-group temporal biases [12,13,14]. Already, the use of test batteries (i.e. combinations of two or more different tests or paradigms in one experimental procedure) for animal behavioural studies has been recommended to facilitate the detection of diverse genuine anxiety-related indices. Additionally, this serial arrangement would also minimize the numbers of laboratory animals needed since only one experimental subject, instead of three, would be run sequentially on a set of three different test apparatuses, similar to the method adopted in a previous study [15] - with a modification of reducing inter-test latencies to zero.

This research is being undertaken to determine the sensitivity of the test apparatuses and the reliability of the serial multi-test protocol in mice exposed to vehicle and experimentally relevant doses of a standard anxiolytic drug.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Experimental animals

Healthy male Swiss Albino mice (17-23 g weight) kept under good laboratory practices were used for the study. Twenty-four hours before the start of the test, all the mice were inspected and mice with any gross deformity or exhibiting difficulty in mobility were removed.

2.1.2 Study arena/test batteries

The test arena was a large room partitioned into equal-sized cubicles each of which had a test battery illuminated by a 100-lux fluorescent bulb hung 80 centimetres above the test apparatuses with light reflections kept to the minimum by the opaque nylon partitions (Fig. 1).

2.1.2.1 The open-field test apparatus

The open-field behavioural test apparatuses are transparent rectangular open plastic boxes (Fig. 1). Each device is 30 cm in height, 60 x 40 cm at the top and 58 x 38 cm at the base. The floor is divided into 4 rolls of 6 squares each, with the central 4 squares designated as the centre zone while the rest of the squares constitute the peripheral zone. The open-field apparatuses are supported on 50-cm high flat-topped stands.

2.1.2.2 The elevated zero-maze device

The elevated zero-maze devices each consist of a 5-cm circular runway that has an external diameter of 61 cm (Fig. 1). The 4-mm thick polyvinyl glass runway has fastened to its entire length an overlay of a thin layer of fine glass pebbles. The runway is divided into four equal segments – two closed segments interspersed by the open segments. The zero-maze device is supported on an 80-cm tall black-painted 4-legged stand made of polyvinyl cellulose.

Fig. 1. Serial test battery set-up with a mouse in-situ

2.1.3.3 The staircase test apparatus

The staircase device is made of light lemon-coloured transparent open glass boxes whose height, length and width are 30 cm, 45 cm and 10 cm, respectively (Fig. 1). On the floor is a stair of 5 steps; the most proximal of which is 2.5 cm high and the most distal step being 12.5 cm high. All the steps are 7.5 cm deep and 10 cm wide and have fastened to their surfaces thin-layer overlays of fine glass pebbles to facilitate mouse mobility. Each staircase apparatus is supported on a stand about 85 cm from the floor.

2.2 Behavioural Test Procedure

Thirty minutes following intraperitoneal injection of 10 ml/kg distilled water, 0.5 mg or 1.0 mg/kg body weight, randomized groups (n=8) of mice were each serially exposed to the open field, zero mazes and staircase similar to the method adopted in a previous study [15] - with a modification of nil inter-test latencies. Each trial was always begun with the open, then, the zero-maze and finally the staircase apparatus with each mouse allowed to freely explore each test for 5 minutes. All behavioural tests were videotaped and visually monitored.

Feacal pellets and/or urinations were counted and recorded against respective test subjects at the end of each behavioural test before the test apparatuses were cleaned up.

2.3 Statistical Analysis

Anxiety parameters were expressed as mean ± SEM using analysis of variance (ANOVA) with

inter-group differences analysed using Turkey's test of multiple comparison test. *P*-value < 0.05 was considered as significant.

3. RESULTS

3.1 Open Field Test

While 0.5 mg/kg diazepam dose significantly (P < 0.05) altered all the three anxiety parameters in mice, 1.0 mg/kg diazepam treatment only caused significant (P < 0.05) attenuation in the mean frequency of feacal pelletisation and/or urination in mice (Table 1).

3.2 Elevated Zero Maze Test

Compared to distilled water-treated mice, groups of mice treated with 0.5 mg and 1.0 mg diazepam per kg body weight exhibited significantly (P < 0.05) lesser number of stretch attend postures and feacal pellets. They also exhibited, albeit insignificantly (P < 0.05), greater percentage open segment time and open segment re-entries. On this test, compared to 1.0 mg/kg diazepam treatment, 0.5 mg/kg diazepam dose exhibited superior anxiolytic activity in % OST and OSR while sharing about equal activity in SAP and frequency of urine/feacal pellet excretion (Table 2).

3.3 Staircase Test Results

Mouse groups treated with 0.5 mg and 1.0 mg diazepam per kg body weight displayed significantly (P < 0.05) lesser mean rears of 6.75±1.90 and 5.00±1.02, respectively, compared to untreated mice with 34.63±13.39 mean rears. No significant differences were observed in the number of stairs climbed amongst the treatment groups (Table 3). On this test, diazepam exhibited a dose-dependent reduction in rearing frequency and attenuation of urine/feacal pellet excretion compared to distilled water treatment (Table 3).

4. DISCUSSION

The three rodent behavioural models i.e. the open field, zero-maze and staircase tests are among the most widely used *In-vivo* anxiety efficacy paradigms in preclinical drug discovery due to their sensitivity to rodent anxiety-related behaviours [16,17,18,19]. The advantages of serial deployment of these murine anxiety tests have also been previously highlighted [9,14,15, 16,20].

Table 1. Mean anxiety indices of mice subjected to open field test following treatment with distilled water, 0.5 and 1.0 mg/kg diazepam

Exp. group (n=8)	% Centre zone time	No of rears	Feacal pellets/ urine
Distilled Water	4.7±0.84	32.50±7.56	1.13±0.45
Diazepam (0.5 mg/Kg)	11.00±2.49*	18.88±4.67*	0.25±0.16*
Diazepam (1.0 mg/Kg)	8.04±1.21	22.00±6.36	0.25±0.15*

*Data were entered as mean ± S.E.M of mice. * = value significant (p < 0.05)*

Table 2. Mean anxiety indices of groups of mice subjected to the zero-maze test

Exp. groups (n=8)	% OST	SAP	OSR	Feacal P/UF
Distilled water	20.08±1.97	4.50±1.12	9.00±1.73	0.25±0.15
DZ (0.5 mg/kg)	26.45±4.64	0.88±0.28*	13.75±2.45	0.00±0.00*
DZ (1 mg/kg)	22.88±3.67	0.75±0.29*	15.50±2.64	0.00±0.00*

*Data expressed as mean ± S.E.M of mice. * = value significant (p < 0.05)*
DZ = Diazepam; % OST = percentage open segment times; SAP= stretch-attend postures;
OSR = open segment re-entries; Feacal P/UF = feacal pelletation/urination frequencies;

Table 3. Mean anxiety indices of groups of mice subjected to the stair-case test

Exp. group	Rears	Stairs climbed	Feacal pellets/ urine
Distilled Water	34.63±13.39	23.96±1.82	0.63±0.25
0.5 mg Diazepam/kg	6.75±1.90*	23.00±2.67	0.25±0.15*
1.0 mg Diazepam/kg	5.00±1.02*	22.38±1.77	0.00±0.00*

*Data were entered as mean ± S.E.M of mice (n=8). * = value significant (p < 0.05)*

Percentage centre zone time (%CZT), rears, and feacal pellets/urine - evaluated in this study are key anxiety indicators in the open-field test. Percentage CZT reflects the proportion of the test duration spent in the centre of the test tool. The more the %CZT, the less the anxiety levels in a particular mouse. A rear or rearing event occurs when the test animals stand erect on their hind limbs with their forelimbs completely in the air or hanging on the wall of the open field. The frequency of rearing increases with increased anxiety levels. Increased feacal palletization/urination is viewed to indicate increased emotionality in rodents [21].

The finding of greater thigmotaxis (reduced central zone time/exploration), increase in rears and in the number of feacal pellets excreted in untreated mice compared to diazepam-treated ones in this test implies this open-field behavioural assay/apparatus was sensitive to rodent anxiety behaviours and the anxiolytic effects of diazepam – a classical anxiolytic.

Percentage open segment time (%OST), open segment re-entries (OSR), stretch-attend postures (SAPs) and feacal pellet/urine excretion are well-established rodent anxiety indices in the elevated zero-maze test [22,23]. Percentage open segment time is defined as the fraction of the total test duration spent on the open segment portion of the elevated zero-maze. OSR refers to the number of returns a mouse makes to the open segment of the maze within the test duration and increased OSR is said to indicate anxiolysis. A stretch-attend posture is a risk assessment behaviour of rodents and it involves alternating episodic stretching and recoil of the animal's entire length while lowering and keeping its trunk to the floor. The observed decreased mean %OST and OSR, and increased mean SAPs and frequency of feacal palletization/urination in the distilled water treated mice in contrast to diazepam-treated mice show the elevated zero-maze test apparatus was sensitive and therefore suitable to measure rodent anxiety-related behaviour and anxiolytic effects of known and putative agents. The finding in this study that treatment with diazepam (a classical benzodiazepine anxiolytic drug) reversed all the anxiety indices observed in untreated (negative control) mice is consistent with the findings in previous studies [24,25]. This finding may confer a predictive validity on this test paradigm/apparatus.

The number of steps climbed, rearing and feacal palletization/urination frequency are the evaluated anxiety indices on the staircase test. On this behavioural assay, only the number of upward steps climbed is used to gauge anxiety levels in rodents. Increased number of steps climbed is thought to imply reduced anxiety and increased exploration in rodents [26].

The observed exaggerated rearing and feacal palletization/urination in the untreated mice and their reversal and total attenuation by diazepam treatments is a pointer to the sensitivity and reliability of this test to measure rodent anxiety and its modulation by anxiolytic drugs. These outcomes are in agreement with the finding of previous studies in which reduced feacal pellet/urine excretion and rears were shown to mirror anxiolysis [26,27,28].

The mean number of steps climbed by the different treatment groups in this test shows no significant ($P > 0.05$) variation. This observation is in agreement with the findings in a previous study [29] whereby extract-treated and benzodiazepine-treated rodent groups exhibited no disparity ($P > 0.05$) in the number of stairs climbed compared to the negative control but had their rears significantly ($P < 0.05$) reduced to half the value obtained in the untreated (negative) controls.

5. CONCLUSION

A perusal of the results from the three individual behavioural paradigms in the test battery indicates high concordance among related anxiety parameters despite the serial testing arrangement. For instance, two anxiety indices that are among the most accepted indicators of rodent anxiety behaviour i.e. open-field% CZT on one hand, and the elevated zero-maze %OST [25] on the other, are largely in agreement in this study. The concordance between behavioural data generated by the serial multi-testing method in this study and those generated by other previous single-test and multi-test protocols [13,14,30] suggests the serial multi-test method adopted in this study is reliable and, therefore, suitable for invoking and detecting murine anxiety-related behaviours.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard written ethical permission has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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