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Research Article
Anxiogenic Effect of Single Swim Session is Reversed by Diazepam in Adult Male Wistar Rats

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Abstract

Background and Objective: Anxiety is a disorder with a high prevalence worldwide that requires further study and the consequences of stressful situations contribute to its development. At the preclinical level, a single swimming session is an intense and inescapable stressor that induces despair and other alterations in hemostasis in rats. This study examined the effect of a single swim session (SS) on the development of anxiety-like behavior and the reversal of the anxiolytic diazepam in the elevated plus maze (EPM) in adult male rats. **Materials and Methods:** Four groups: Control group that received saline (n = 8) and diazepam group (DZP, 2 mg/kg, n = 7) were not submitted to swimming; SS (n = 8) and SS+DZP (n = 8) swam for 15 min 24 h before EPM. Diazepam or saline was i.p., injected 1 h before the EPM test. The variables evaluated were time in seconds, number of entries and percentage of entries into open arms, anxiety index and total entries in EPM, which were analyzed by one-way analysis of variance. **Results:** The SS group spent less time on the open arms and had a higher anxiety index, with no changes in total arm entries compared with the control group (i.p., saline, 1 mL/kg, n = 8). The DZP produced the highest time spent in open arms and the lowest anxiety index in rats without swimming. This benzodiazepine in rats submitted to swimming produced a significant anxiolytic effect compared to the SS group but less than DZP alone. **Conclusion:** These findings indicated that a single 15 min SS session is a potent anxiogenic stimulus in adult male Wistar rats and diazepam blocks this anxiogenic effect. Therefore, the strong stress experience leads to the foresight of working with anxiety-induced animals, impacting basic sciences and conferring perspectives for studying certain types of anxiety with behavioral pharmacology methods.

Key words: Anxiogenic effect, swim stress, diazepam, behavioral pharmacology, elevated plus maze

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anxiety is a disorder with a high prevalence worldwide necessary for further study and the consequences of stressful situations contribute to its development[1]. At the preclinical level, various behavioral tests allow the study of anxiety, for example, Vogel test, open field, light/dark box, hole board, social interaction and elevated plus maze (EPM), among others[2-4]. All of them are carried out with the aim of understanding the brain function underlying the presence of the pathology, as well as detecting new substances with potential anxiolytic effects. Particularly, EPM measures the natural aversion of animals to open and elevated spaces[2] which resembles specific phobia where subjects are anxious about specific environments such as natural environments (i.e., acrophobia or fear of heights) and/or agoraphobia where individuals are anxious about being in open spaces (among other situations), according to DSM-5[5].

The anxiety is developed in response to a threat that is a stressor over which the animal has no control. In this study, the known swimming test was employed, which is used to evaluate immobility time in rats in a pool during a 5 min test at least 24 h after a 15 min pretest session when despair-like behavior is generated[6]. This methodology has been widely used to screen antidepressant substances. There are still some controversies about the meaning of immobility. Antidepressant drugs, more than exerting an anti-despair action, appear to cause a switch from passive coping strategies to active coping strategies to deal with an unescapable situation that is represented by being submitted to swim[7]. A single swim session (SS) produces striking changes in neuronal function. For example, in the lateral septal nucleus which is a forebrain structure that is related to emotional regulation[8], a lower neuronal firing rate occurs in rats submitted to pretest and test swim sessions[9]. The SS also activates the hypothalamic-pituitary-adrenal axis, producing high levels of adrenocorticotropic hormone and corticosterone secretion[10], decreases the activity of neurotransmitters (e.g., dopamine[11], serotonin[12] and γ-Aminobutyric Acid (GABA))[13], decreases the expression of brain-derived neurotrophic factor in young rats[14] and decreases the survival of hippocampal cells[15]. Therefore, a single swim session must produce measurable changes in behavior using an accepted model to explore anxiety.

The relationship between swim sessions and anxiety has been studied. Swimming experiences differentially affect anxiety-like behavior depending on the specific anxiety test used. For example, swimming may not induce anxiety in the light/dark test[16], but it increases social anxiety in male adolescent rats[17]. In 21 days old infant animals, a single swim session produces anxiety-like behavior in the EPM measured after 24 h[18]. Unknown, however, is whether these effects of a single swim session produce comparable actions in adult rats and whether this anxiogenic action may be suppressed by the administration of an anxiolytic drug acting on GABAergic system. Other study in adult rats showed that the anxiogenic effect of pre-test and test sessions of swimming are reverted by chlorpheniramine, through a signaling mechanism of antioxidant defense and neurotrophic support[19]. Therefore, the objective of the study was to evaluate the impact of a single swimming session on the development of anxiety-like behavior and the reversal of the anxiolytic diazepam in the EPM in adult male rats.

MATERIALS AND METHODS

Study area: The present study was carried out in a sound-buffered room with 40 Lux of illumination in the behaviour area in the Neuropharmacology Lab at the Institute of Neuroethology, University of Veracruz, Xalapa, Veracruz, Mexico, from July, 2019 to July, 2021.

Ethics: The experiment strictly followed the Guide for the Care and Use of Laboratory Animals[20] and was approved by the Ethical Committee of the Biomedical Research Institute, National Autonomous University of Mexico (CICUAL ID 259).

Animals: Adult male Wistar rats were obtained from colonies at the local animal breeding unit at the Institute of Neuroethology, University of Veracruz. The study included 31 male Wistar rats (2 months old), weighing 250-300 g. The rats were housed in the animal facility of the same institute and were randomly assigned to four groups: Control (CTRL, n = 8), diazepam (DZP) (n = 7), single swim session (SS, n = 8) and single swim session+diazepam (SS+DZP) (n = 8). The rats were housed in five per cage in acrylic boxes (44 cm length×33 cm width×20 cm height) in the animal room with a 12/12 h light/dark cycle and free access to purified water and food (Teklad Lab Animal Diets, Harlan, Indianapolis, Indiana, USA).

Stress session: Only the SS and SS+DZP groups were subjected to a single swim session. The rats were individually placed in an acrylic cylinder (49×20 cm) that was filled with 25±1°C water. The depth of the water allowed the rats to maintain an upright position to barely touch the bottom of the pool with their hind paws and keep their nose above the water. The rats swam in a single session for 15 min[6]. No behaviors were measured or recorded during this session. The anxiety variables were measured in the EPM 24 h after the single swim session and 1 h after the diazepam injection.

Diazepam treatment: Saline solution (0.9% NaCl) was used as the vehicle and intraperitoneally (i.p.) injected in a volume of 1 mL/kg in rats in the CTRL group and SS group. Diazepam (Valium 10, Hoffman-Roche, Basel, Switzerland) was dissolved in the vehicle and injected at a dose of 2 mg/kg (i.p.) in the DZP and SS+DZP groups.

One hour after the injection, the rats were individually subjected to the EPM. All procedures were performed during the light period between 11:00 am and 2:00 pm.

Elevated plus maze: The EPM was constructed of wood, formed by four opposite arms in a plus configuration. The two open arms (50 cm length × 10 cm width) were painted white. The two closed arms (50 cm length × 10 cm width, with 40 cm high walls) were painted black. The EPM was elevated 50 cm above the floor. The rats were individually placed in the center of the EPM, facing an open arm and were free to explore the apparatus for 5 min.

The following anxiety variables were evaluated: Time spent (in seconds) on the open arms, number of entries into the open arms to estimate percentage of open arm entries and the anxiety index (AI)[21] calculated as the number of entries into the open arms and time spent on the open arms relative to the duration of the test (in seconds) and total number of entries:

$$AI = 1 - \frac{\frac{\text{Open arm time}}{\text{Test duration}} + \frac{\text{Open arm entries}}{\text{Total number of entries}}}{2}$$

The higher the AI, the greater the anxiety state of the rat. A total number of arm entries (open arms+closed arms) was considered a measure of locomotor activity. The apparatus was cleaned with a 15% ethanol solution after each animal was tested. Two blind independent observers measured the behavioral variables using *ex profeso* software named SDLCONX v1.0 to record the number and time (in seconds) of each evaluated behavioral variable until >95% agreement was reached among observers.

Statistical analysis: For a normal data distribution, One-way Analysis of Variance (ANOVA) was applied. Values of $p \leq 0.05$ in the ANOVA were followed by the Student-Newman-Keuls *post hoc* test. The data were analyzed using Sigma Plot 12.0 software (Systat Software, Chicago, Illinois, USA). The results were presented as the Mean±Standard Error of the Mean.

RESULTS

The time spent on the open arms was significantly different among groups ($F_{(3,27)} = 20.812$, $p \leq 0.001$). The *post hoc* test revealed that the SS group spent significantly less time on the open arms in comparison with CTRL, DZP and SS+DZP groups. Diazepam by itself produced almost a double-fold increased open arm time compared to the CTRL group, while DPZ in the stressed animals submitted to swimming (SS+DZP) produced a higher open arm time which was significantly different from SS group but significantly lower compared to only DZP group (Fig. 1).

The percentage of entries into the open arms was also significantly different among groups ($F_{(3,27)} = 25.928$, $p < 0.001$). The DZP group and SS+DZP group exhibited more open-arm entries than the CTRL and SS groups. A trend to reduce the percentage of open-arm entries was observed in the SS versus the CTRL group ($p = 0.06$) (Table 1).

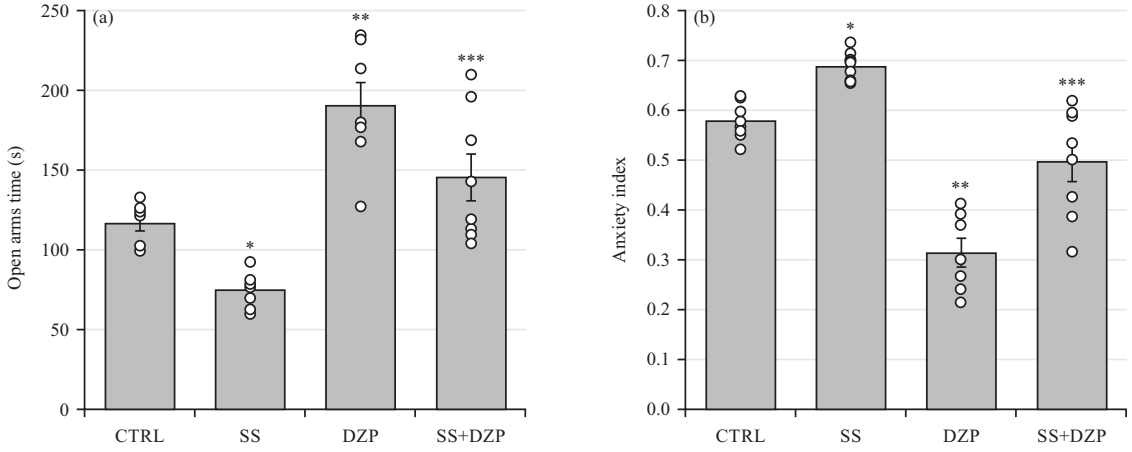

Fig. 1: Anxiety variables in the EPM 24 h after the swim session, (a) Open arms time and (b) Anxiety index
*p<0.01 vs all groups, **p<0.01 vs CTRL and ***p<0.01 vs DZP

Table 1: General locomotion in EPM is not altered by the anxiogenic effect of swim

Group	Entries into the open arms (%)	Total number of entries
CTRL	45.3±2.6	17.0±1.9
SS	37.3±2.3*	17.0±2.2
DZP	73.6±3.8**	15.1±0.9
SS+DZP	52.0±3.0	22.2±1.6

CTRL: Control group, SS: Single swim session group, DZP: Diazepam group, SS+DZP: Single swim session+diazepam group, *p<0.01 vs SS+DZP and **p<0.001 vs all groups

The AI significantly differed between groups ($F_{(3,27)}$ = 36.629, p<0.001). Consistent with the time spent on the open arms, the swim session produced a higher AI versus all groups (i.e., SS group versus CTRL, DZP and SS+DZP), revealing anxiety-like behavior. Diazepam in rats without swimming produced the lowest AI compared with all groups. The anxiolytic effect of diazepam was also observed in SS+DZP compared to the CTRL group but was not equal to DZP alone (Fig. 1). No differences were observed in the total number of entries, only a trend in the SS+DZP group to increase this variable ($F_{(3,27)}$ = 2.861, p = 0.055) (Table 1). The number of total entries was similar between groups.

DISCUSSION

Stress activates emotional responses that occasionally produce non-adaptative responses, leading to anxiety disorders. Anxiety may be induced by chemical stressing procedures like pentylenetetrazol[22], lipopolysaccharide[23] or ethanol withdrawal[24], among others. A more ethological model is represented by the delivery of auditive stimulus resembling predatory sounds[25], social defeat[26] or psychogenic stress[27]. So, preclinical research in mice or rats uses stressors to model some clinical features of anxiety-like behavior like aversion to open and elevated spaces as could happen in natural environmental situations. For this reason, the objective of the present study was to evaluate the effect of a single 15 min swim session on anxiety-like behavior in adult male rats using the EPM.

The EPM has been extensively used to study anxiety-like behaviors and the effects of anxiolytic drugs in male and female adult rats. In this test, anxiety is generated by rodents' unconditioned (innate) fear of heights and open spaces, leading them to naturally wander into the closed and confined space of the closed arms[2]. Greater anxiety is interpreted as a lower exploration of open arms. Its validity as a model for human anxiety has been questioned for several reasons[28]. However, EPM continues to be useful for studying some basic and specific aspects of the neurobiology of anxiety[29]. In present study, observed anxiety-like behavior induced by a stressor like swimming in Wistar rats after 24 h of being exposed, which increases the anxiety evaluated in the EPM, a model that evaluates the anxiety produced by exposure to open and elevated spaces, i.e., agoraphobia and acrophobia. Another study that measures another type of anxiety, i.e., in the social interaction test, found that two 10 min swim sessions do not change the overall spectrum of social anxiety-like behavior in adult male Sprague-Dawley rats, suggesting that this stressor may be less demanding for adults when compared with adolescents[14]. The Sprague-Dawley strain of rats is more appropriate for social tests because of their characteristic motivation to interact with social stimuli[30], whereas the Wistar strain is more suitable for testing in the EPM based on validated findings of exploring novel non-social spaces[2].

Other studies have explored relationships between pretest and test swim sessions and anxiety-like behaviors. Interesting and complex data have been reported when the EPM test is performed first to classify high and low anxiety and then the swim test is performed later[31,32]. By contrast, rats classified with high- or low-immobility in swim tests submitted to chronic unpredictable mild stress do not differ in their anxiety measured in experimental behavioral models[33]. Therefore, the study focused on conducting the swimming session first to induce a level of anxiety equivalent to a pathological condition, rather than simply basal anxiety and subsequently measure it in the EPM. The literature shows a poor relationship between immobility in the swim test and anxiety in novel environments, although results may depend on the kind of coping (active or passive) followed by the experimental subject[34].

The anxiolytic actions of diazepam have been tested in the EPM[35,36] and other tests. The GABAkines are positive allosteric modulators of $GABA_A$ receptors. Diazepam is a well-known member of this group of drugs that are used to manage diverse clinical conditions, such as anxiety and epilepsy, among others[37]. Diazepam similarly reduces immobility in the swim test as the tricyclic antidepressant imipramine[38] and increases the time spent on the open arms in stressed animals through actions on $GABA_A$ receptors[39]. This was consistent with that was obtained in the present study; the administration of diazepam in the

positive control group produced the expected anxiolytic effect by increasing the time in the open arms and reducing the anxiety index. However, neither the swimming session nor the administration of diazepam produced changes in locomotion. Locomotor activity in the EPM is usually measured by tracking the movement of the animal as it explores the maze. This can be done by counting the total number the entries into and exits from, the open and closed arms[40]. So, the lack of difference between groups indicates that locomotion was unaltered by the single swim session and that the anxiolytic dose of diazepam (2 mg/kg, i.p.) did not reduce locomotion to influence the EPM results.

Regularly, SS, EPM, open field and other well validated behavioral tests, are included in a battery of tests for screening substances with potential anxiolytic or anti-despair actions[41,42]. A battery of tests was not performed in this study, but if SS is included then it must be in the last place to avoid an effect on any subsequent behavioral test. The present study highlights the use of SS as stressor producing anxiety, before the administration of the experimental compound, in this case the well-known anxiolytic drug, diazepam.

The measurement of physiological markers of the stress response would have provided more information about the impact of swim stress 24 h later. Nonetheless, the direct effect on behavior was observed in a very sensitive model of anxiety, the EPM. The sample size (n = 8/group) was sufficient to detect both anxiogenic and anxiolytic effects in behavioral tests without compromising the statistical analysis. This sample size was based on Russell's 3Rs (Reduce, Refine, Replace) and our previous studies that included groups of n = 7 or 8 rats[43-45]. Whether different times of swim sessions are also sufficient stressors to exert an anxiogenic effect in the EPM 24 h later and to explore the effect of swimming in female rats were some aspects to further study. The use of additional tests of anxiety could provide information on other types of anxiety-like behaviors induced by swimming, so the contribution in this short communication serves to concrete rather than to generalize in the study of the complex construct that is anxiety behavior.

CONCLUSION

The swim session (i.e., an inescapable stressor) exerted anxiety-like behavior in adult rats in the EPM 24 h later. This finding is useful for discerning the impact of a single swim session when subsequently assessing behavior in animal models of stress-related behaviors. The effect of diazepam revealed a likely GABAergic mechanism that is related to swim-induced anxiety-like behavior in adult male Wistar rats.

SIGNIFICANCE STATEMENT

It is unknown the impact of a swim session on anxiety-like behavior in adult rats after a separate time interval. The contribution lies in considering that the swim effect represents a source of anxiety, becoming a confounding variable in behavioral pharmacology research. The study aimed to explore the anxiogenic response to swim sessions and the pharmacological effect of the benzodiazepine diazepam to attenuate this response. The main result was that acute swim stress is sufficient to induce anxiety-like behavior measured in the elevated plus maze 24 h later, an effect reverted by diazepam through a likely GABAergic mechanism, which suggests running first anxiety tests with this methodology.

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