Assiut Vet. Med. J. Vol. 45 No. 89, April 2001

Dept. of Animal Hygiene,
Fac. Vet. Med., Assiut University.

ELEVATED PLUS-MAZE TEST SENSITIVELY REFLECT DIFFERENCES IN ANXIETY LEVELS AFTER EXPOSURE TO VARIOUS STRESSFUL CONDITIONS IN RATS

(With 1 Table and 4 Figures)

By

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(Received at 1/2/2001)

اختبار قياس التوتر هو أكثر حساسية لتحديد مستويات القلق في الفئران بعد تعرضها لظروف مختلفة من التوتر

مديحه درويش – تشابا نياكش – لايوش كورانى

أجريت هذه الدراسة على عدد (١٠٨) من ذكور الفئران " نوع ويستر" وقسمت الفئران إلى ستة مجموعات (١٨ فأر لكل مجموعة) واعتبرت المجموعة الأولى كضابط للتجربة، أما المجموعات الثانية وحتى السادسة فقد تعرضت لمدة ساعة واحدة إلى حالات مختلفة وحادة من التوتر مثل : الحقن بمحلول فسيولوجي ٢% ، أو لكريات زجاجية ، أو حقنت بالمحلول الفسيولوجي ثم تعرضت لكريات زجاجية ، أو تعرضت لجرعة كهربائية في القدم ، أو تعرضت لعدد من الفئران حديثة الولادة وبعد ذلك قسمت كل مجموعة إلى قسمين ، أستخدم القسم الأول (عدد ٩ من الفئران) لقياس درجات التوتر ، أما القسم الثاني (عدد ٩ فئران) فقد أخذت منه عينات دم لقياس تركيز هرمون " الكورتيكوستيرون " وأظهرت هذه الدراسة النتائج الاتية : ارتفاع تركيز هرمون " الكورتيكوستيرون " في بلازما دم جميع الفئران التي تعرضت للتوتر وكان هذا الارتفاع معنويا عند مقارنته بالمجموعة الضابطة ، كما لوحظ أيضا عدم وجود فروق معنوية في مستويات الهرمون فيما بين المجموعات التي تعرضت للتوتر.، بعد قياس مستويات القلق ، وجد زيادة معنوية في مستويات القلق لدى جميع الفئران بعد تعرضها للتوتر إذا ماقورنت بالمجموعة الضابطة ، كما لوحظ أيضا فروق في مستويات القلق بين المجموعات التي تعرضت للتوتر ، وكان أكثر المجموعات توترا تلك التي تعرضت لتوتر مزدوج (المجموعة الرابعة) ، وأقلها اضطرابا تلك التي حقنت بالمحلول الفسيولوجى (المجموعة الثانية). لذلك يمكن القول بأن مستويات القلق تتأثر بشدة بنوع التوتر التي تتعرض له الفئران ، كما وأن جهاز قياس مستويات القلق هو أكثر حساسية ودقة لقياس مستويات القلق لدى الفئران بعد تعرضها لحالات حادة من التوتر .

SUMMARY

Male wister rats were exposed to acute stressful conditions to measure plasma corticosterone (CORT) concentrations and to detect the anxiety levels in response to stress. Stressed rats showed significant elevations of CORT concentrations as compared to unstressed (control) rats. No significant differences were observed between stressed rat groups. In the plus-maze apparatus, stressed rats exhibited a higher levels of anxiety as judged by their significantly lower time spent in the open arms as well as significantly decreased latencies to first entery into the open arm of the plus-maze apparatus. Significant differences in the anxiety levels were observed between stressed rat groups with the hightest levels of anxiety were observed in rats exposed to double stressful stimuli (saline injection followed by exposure to marbles), while saline-injected rats exhibited lower levels of anxiety. Taken togerther, these results provide an additional support for the use of elevated plus-maze as a sensitive measurement of anxiety in response to acute stress in rats.

Key Words: *Plus-maze, Anxiety, Stress, CORT levels, Rats.*

INTRODUCTION

A great variety of stressors have been used in research aimed at description and analysis of physiological and behavioral components of stress response. The major component of the physiological response to stress is activation of the hypothalamo-pituitary-adrenal mechanism. Corticotropin- releasing hormone (CRH) of the hypothalmus is the peptide closely concerned with stress. The amount of CRH in the brain is increased by stress. CRH stimulate secretion of ACTH from the anterior pituitary which in turn influence the secretion of adrenal corticosteroids. Thus, acute exposure to a variety of stressors results in enhanced secretion of ACTH and adrenal corticosteroids. Infusions of CRH into the brain induces anxiety-like behavior (Baldwin et al., 1990), while blocking its action, reduces the fear or anxiety. Consequently, fluctuations in plasma levels of corticosteroid could be used as an index of the emotional state in response to stress.

Hennessy and Levine (1978); Hennessy et al., (1979); found that when mice and rats exposed to 3 increasingly unfamiliar environments (low intensity stress) for 15 min. showed 3 corresponding elevations in plasma corticosterone levels. In contrast, they demonstrated that when a rodent exposed to an intense psychological stimulus followed by

additional stimulation of sever intensity, the mean plasma corticosterone levels did not show a significant elevation in response to the additional stimulus. Furthermore, earlier studies (Friedman and Ader, 1967; Ader, 1970; Bassett et al., 1973) reported that plasma corticosterone was significantly elevated in rats after receiving electric foot-shock, however, when these animals have been subjected to increased intensities of electeric shock, the plasma corticosterone levels were not significantly elevated. The above mentioned studies (Friedman and Ader, 1967; Ader, 1970; Bassett et al., 1973; Hennessy and Levine 1978; Hennessy et al., 1979) were failed to fined significant differences in the plasms levels of CORT following exposure of rats to ranges of sever stimuli, and reported that corticosterone response is an insensitive measure of emotional state of these animals. One possible explanation of these findings is that adrenal glands produced the maximal levels of corticosterone. However, it can be suggested that variation in the emotional state of rats exposed to different forms of acute stress could be sensitively measured in the plus-maze test. The present investigation examined this possibility by exposing rats to different forms of acute stressful conditions to detect, a) the changes of plasma corticosterone; b) anxiety levels measured in the plus-maze apparatus and c) to compare the changes in plasma CORT concentrations with the levels of anxiety in response to these stressful stimuli.

Several stressful events, known to stimulate pituitary-adrenal mechanism, have been used to examine the variations in plasma CORT levels in relation to stress. Injection of hypertonic (2%) saline solution is frequently used as acute physiological stressor. Acute stressful conditions were also demonstrated in rats after exposure to novelty, such as glass marbles (Pinel and Treit, 1983; Broekkamp et al., 1986) or rat pups (Vogt et al., 1984). Electric shock when delivered to the rat feet elicit a state of intense physical stress (Levine, 1962).

In the present experiment the following forms of acute stressful conditions were applied as puplished elsewhere; glass marbles (Pinel and Triet, 1983); saline injection (Ward et al., 2000), electric foot shock (Levine, 1962); and exposure to rat pups (Vogt et al., 1984).

MATERIALS and METHODS

Experimental Animals

Outbred male rats of Wistar strain were used in these experiments. All rats (N=108) were housed in metal top-loading

polyethylene cages (40 × 23 × 24 cm) with sawdust bedding and the environmental temperature was maintained at approximately 22°C. Food and water were available ad libitum, and lights were maintained on a 12-hr light/dark cycle (lights on at 06:00 am). All experimental procedures conformed with the law of animal welfare.

Experimental procedures

Animals used in the present experiment were divided into 6 groups and each group (N=18) was assigned to a specific treatment condition. Rats of all experimental groups were housed individually (one/cage; 28 × 20 × 15 cm) for 3 days before treatment. Animals in each group were exposed to one of six experimental conditions, then subdivided into 2 subgroups, the first subgroup (N=9) was used for testing the anxiety levels in the plus-maze test, while the second subgroup (N=9) was used for measuring the mean levels of plasma corticosterone (mention below)

Group 1: (Controls)

Rats in this group were left undisturbed and used as controls. The anxiety levels were measured in the plus-maze test, while blood samples were obtained to measure the control level of plasma corticosterone.

Group 2: (Saline injected)

Rats were injected intraperitoneally (i.p) with 2% saline solution, and after 60 min of injection, the animals were tested for anxiety or CORT concentration.

Group 3: (Exposed to marbles)

Each individual rat was exposed to 5 marbles for a period of 1 hr. The marbles were small round glass balls of white colour and measured 12 mm in diameter. The marbles were placed in close contact in the middle of the cage on 5 cm layer of sawdust. After one hour of exposure to marbles, rats were tested for anxiety or CORT levels.

Group 4: (Saline → Marbles)

Rats of this group were injected intraperitoneally with 2% saline, then exposed to marbles similarly to those in group 3, and after one hour of exposure to marble, animals were tested either in plus-maze test or blood sampled for CORT concentrations.

Group 5: (Electric Shock)

Rats were placed individually in well illuminated (100 Watt) shock chamber (30 × 30 × 30 cm), then received unavoidable electric foot-shock of 0.8 MA (milli-ampere) for 15 seconds followed by 15 seconds interval, this shock repeated 4 times. The shock was delivered to the animal feet through a grid floor and the total time of exposure was 60

(4×15) seconds. The rats were then removed from the shock chamber and tested in plus-maze or blood sampled for CORT levels.

Group 6: (Exposed to rat pups)

Animals of this group were exposed individually to the presence of rat pups three rat pups (3-5 days old) were placed on the sawdust bedding at one corner of the home cage of each individual rat for a period of one hour. After such exposure, rats were directly tested for their anxiety state or blood sampled for plasma levels of CORT.

Elevated plus-maze test (Pellow et al., 1985; Rodgers and Cole, 1994)

The anxiety levels of rats in all experimental groups were measured in the plus-maze apparatus. The elevated plus maze (Fig. 1) consists of four arms, each 51 cm long, arranged in the shape of a plus sign, and elevated 50 cm off the ground. Two opposite arms were open platforms having no walls, but having a 0.5 cm rim, whereas the other two arms were closed, with 36 cm-high walls. There is a central square measuring 10.8 × 10.8 cm. The entire plus maze was made of plywood, sanded smooth and painted a flat black.

On the day prior to testing, animals were placed in separate, novel cages in the testing room to habituate the animal to that aspect of the test procedure. On the day of testing, animals were again placed in novel cages and brought into the test room. About four animals were tested per day, with all the animals being brought into the test room and tested in succession. Testing occurred between 10.00 a.m. and 14.00 h p.m., during the light phase of the daily cycle.

The test was conducted under four 25-Watt red incandescent bulbs positioned symmetrically 1.5 m above the plus-maze. A video camera was mounted vertically above the maze, and the behavior was scored by means of a monitor and computer keyboard.The experimenter used to set on an elevated seat 0.5 meters above one arm of the maze and recorded behaviors, blined to the status of the animal.

The following parameters were calculated, (i) the percentage of time spent in the open arms [open time/ (open + closed time) X 100], (ii) the latency to first entry into an open arm. In this test, the greater the time spent in the open arms, as well as the faster the entry into an open arm, reflects lower anxiety levels.

Blood sampling and hormonal assay

Blood samples were collected in EDTA-coated, precooled 1.5 ml tubes. The samples were kept cold until and throughout centifugation (3, 000 rpm for 20 min. at °C). Plasma was extracted, placed in precooled sample vials and frozen (-20 °C) until radioimmunoassy for CORT.

Specific radioimmunoassays (RIA; ICN Biomedial, Costa Mesa, CA) were used to assay corticosterone CORT), following the manufacturer's instructions, except that all samples and reagents were used at half the recommended volumes.

Statistical analysis:

The results are expresssed as mean ± SE of the mean. Evaluation of plasma CORT and behavioral observations were made by ANOVA, which was followed by Student-Newman-Keul test for multiple comparison (Glantz, 1997).

Statistical P-values are calculated by post hoc-t-test after ANOVA. The program sets minimal levels of significance at $P< 0.05$.

RESULTS

At the behavioral levels, it has been demonstrated that rats were capable of displaying diverse defensive reaction in response to the external threats or stressors (saline injection, marbles, electric shock or rat pups). Such behaviors classically comprise exploration of the novel, fear of the stimulus and bury dangerous object (burying behavior) due to the state of hightened arousal in stressed animals.

Hormonal Assay:

As shown in (Table 1 and Fig. 2) blood plasma levels of CORT in stressed rat groups (2-6) were significantly ($P<0.05$) increased as compared to control animals. Although animals of the stressed groups exhibited a higher levels in their plasma CORT reaching about 2 fold than those measured in control rats. Statistical analysis failed to show significant differences in mean CORT values between the treated rat groups.

Table 1: Showing plasma concentration of corticosterone in control and stressed rat groups.

	Corticosterone (CORT)
	Mean ± SE
Control	85.03 ± 9.29
Saline	165.69 ± 14.06
Marbles	160.78 ± 13.39
Saline + Marbles	167.43 ± 11.97
Electric Shock	170.50 ± 8.82
Exposure to pups	172.50 ± 10.06

When additional stimulation in the form of saline injection was followed by exposure to marbles (group 4), the mean CORT value did not significantly increased when compared to those rats received either saline injection (group 2), or exposed to marbles (group 3).

Anxiety test:

As can be seen in (Figs. 3,4) the percentage of total time spent in open arms as well as latencies to first entery into an open arms of the maze were measured in controls and stressed rats. Stressed rats which exhibited significant ($P<0.05$) higher levels of plasma CORT (groups 2-6) showed an elevation of their anxious state as judjed by their significantly ($P<0.05$) lower time spent in the open arms and significantly ($P<0.05$) increased latencies to first entery into an open arms of the plus-maze as compared to control rats. Among the stressed rat groups (2-6), the anxiety levels were significantly ($P<0.05$) different in correspondance to each specific treatment. Rats in group 3, which received double acute stressful stimuli showed the highest levels of anxiety as indicated by the significant ($P< 0.05$) decreased in the total time spent in the open arm of the maze, as well as significant ($P<0.05$) increase in latencies to first entry into an open arm however, those injected with saline exhibited the lowest levels of anxiety among the stressed rat groups.

Moreover, rats which injected with saline followed by exposure to marbles (group 4) showed a significant ($P<0.05$) increased in their anxiety levels when compared to those injected with saline (group 2) or exposed to marbles (group 3).

As shown in (Figs. 4) animals of the treated groups (2-6) showed a significant ($P<0.05$) lower latencies to enter into the open arms of the maze than did rats of control group. In addition, a significant ($P<0.05$) lower latencies to enter into open arm was detected between all stressed rat groups.

DISCUSSION

The response of an organism to aversive stimulation appears to reflect adaptive changes to meet environmental demands. It is well documented that acute exposure to various types of stress resulted in an increased secretion of CRH, which stimulate the pituitary gland to release ACTH resulting in increased release of adrenal corticosteroids (Dallman and Jones, 1973 a.b; Basset and Cairncross, 1975). So, increased concentration of plasma corticosterone is a generalized and central reaction to all stresses (Selye, 1978). Beside the hormonal

response to stress, there is also an imoprtant behavioral response. For example, a whole range of adaptive behaviors are initiated, including the onset of a state of anxiety. The behavioral response to acute sever stress was found to include depressed exploratory behavior, reduced food intake and increased anxiety (Kennet et al., 1986). Yet with time or even with repeated stress (chronic stress) behavior return to normal, indicating adaptation (Stone and Platt, 1982; Stone et al., 1986). So, like the physiological responses, the emotional adaptive changes made to meet the environmental demands. So that, stress is considered to be a causal factor in producing anxiety (Inglis and Fibiger, 1995; Hogg, 1996). In addition, it has been found that fluctuations in corticosterone levels are used as an index of the anxiety state related to stress (Coover et al., 1971; Smith, 1973; Hanson et al., 1976; Veronickos-Danellis and Heybach; 1978). Consequently, exposure of an animal to different conditioned stimuli can produce anxiogenic- or anxiolytic-like effects which can be measured reliably in the elevated plus-maze test.

The plus-maze test was validated behaviorally, physiologically and pharmacologically for identification of anxiolytic and anxiogenic behavior in the rat (Pellow et al., 1985). In rats, an elevation of the anxious state (anxiogenic behavior) was associated with observation of significantly reduced time spent into the open arms as well as significant increased latency to first entry into an open arms of the maze. In contrast a reduction in the anxious state (anxiolytic behavior) was shown to be correlated with significant increase in time spent in the open arms as well as reduced latency to first entry into an open arm (File, 1992; Rodgers and Cole, 1994).

The present results indicated that rats exposed to various ranges of aversive stimului (saline, marbles, saline → marbles, electric foot shock and rat pups) showed a significant higher mean of plasma corticosterone levels if compared to control rats. Also no significant difference in CORT values was observed between treated groups. Moreover, when additional stimulation in the form of exposure to marbles was proceeded by an intense stimulation in the form of saline injection (group 4), the mean plasma levels of CORT did not show an additional elevation in respone to the second stressor.

These results indicating that, pituitary-adrenal responsiveness (CORT level) to these acute stressful conditions, particularly the double-stressed condition, seemed to be quantitatively equal. The most obvious explanation for the lack of selective correspondance of CORT response to these different stressful conditions is that acute (sever) stressors

tended to produce the maximal secretion of corticosterone (Hennessy et al., 1979) on one hand. On the other hand, many other brain peptides are associated with acute stress (vasopression, cholecystokinin and Neuropeptide – Y) to alter other body functions (apetite, food intake, mood) but not involved in the process of CORT secretion (DeGoeij et al., 1991; Herbert, 1996).

Moreover, behavioral testing for anxiety levels of the stressed rat groups showed significant differences in emotional states between these different stressful conditions. The anxious state was found at different rates of augmentation among these various forms of aversive stimuli. So, the existence of such significant difference in the emotional state measured by plus-maze apparatus suggested that the behavioral test provide a useful parameter and sensitive measurement of emotional state after exposure to acute stimuli.

The behavioral testing of anxiety levels in the plus-maze apparatus of the stressed rat groups were significantly higher than those of control group. Moreover, the anxiety levels were significantly different between the treated groups. These results clearly showed the exsistance of differential responsiveness in the emotional state of rats corresponding to different stressful condition.

These differential effects of stress on the anxiety levels of stressed rats could not be contributed to the flactuations in plasma concentration of CORT. Furthermore, this selective responsiveness appears to be largely due to the increase release of vasopressin in response to acute, but not chronic stress in rats (De Goeij, 1991). Vasopressin is a hypothalamic peptide hormone known to potentiate the anxiogenic (increased levels of anxiety) effect of CRH (Herbert, 1997).

In conclusion, these results provide an additional support for the use of elevated plus-maze as a sensitive measurement of anxiety in response to acute stress in rats.

ACKNOWLEDGEMENT

The authers would like to express warm gratitude to all members of the Department of Clinical and Experimetal Laboratory Medicine, Faculty of Health Sciences, Semmelweis University, Budapest, Hungary. Particular thanks to Dr. Mária Sasvári and Dr. Klara Felszeghy, for sharing their knowledge, technical expertise, friendship and humour.

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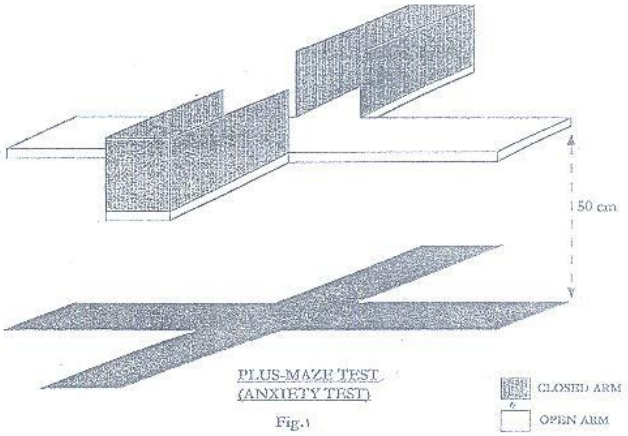
LEGENDS

Fig. 1: Elevated Plus-maze Apparatus.

Fig. 2: Showing plasma concentration of corticosterone levels in control and stressed rat groups. Asterisks indicate significant differences ($P<0.05$) of stressed as compared to control rat groups.

Fig. 3: Showing the time spent into the open arms in relation to total time spent in both closed and open arms of the plus-maze apparatus of control and stressed rats. Asterisks indicate significant differences ($P<0.05$) as compared to control rat groups. Crosses denote significant differences ($P<0.05$) between effects of stress.

Fig. 4: Showing latencies to first entry into open arms of the plus-maze apparatus of control and stressed rat groups. Asterisks indicate significant differences as compared to controls. Crosses denote significant differences ($P<0.05$) between effects of stress.



PLUS-MAZE TEST
(ANXIETY TEST)

Fig.1

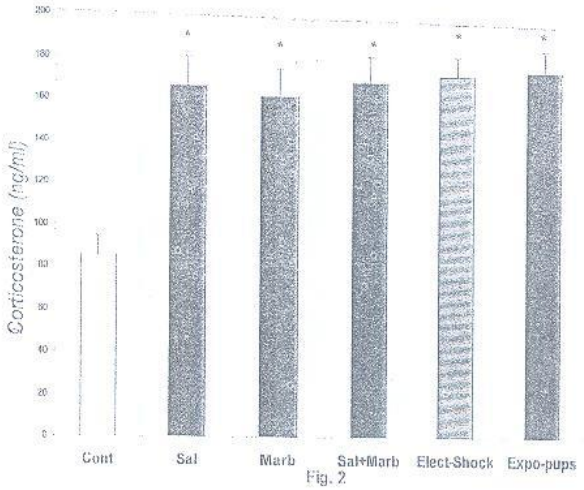


Fig. 2

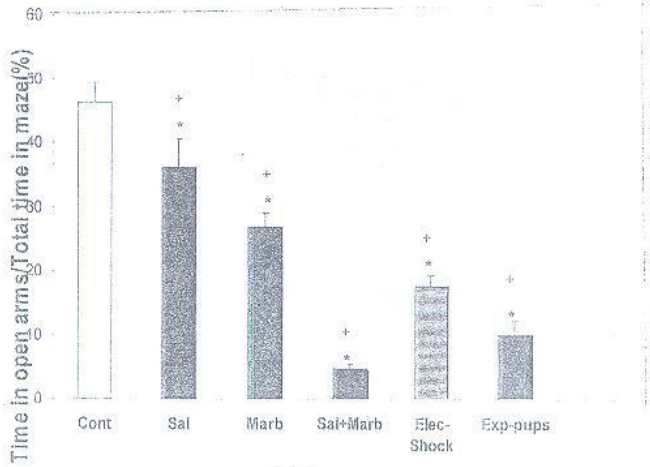

Time in open arms/Total time in maze(%)
60
0
Cont
Sal
Marb
Sal+Marb
Elec-Shock
Exp-pups

Fig. 3

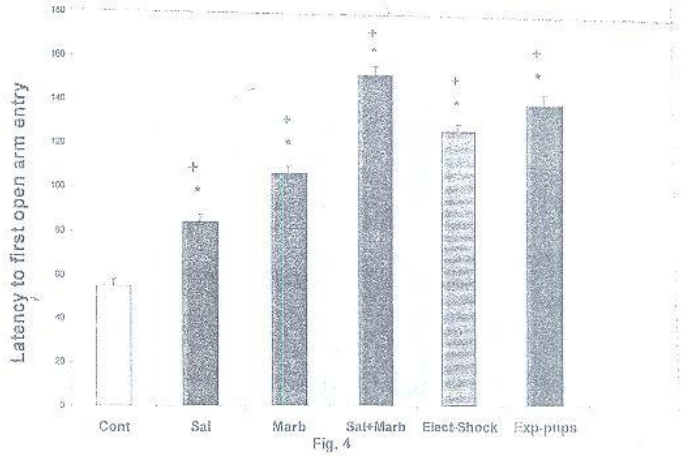

Latency to first open arm entry
Cont
Sal
Marb
Sal+Marb
Elect-Shock
Exp-pups

Fig. 4