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New animal model of emotional stress: Behavioral, neuroendocrine and immunological consequences

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Abstract **This report describes a new model of emotional stress, which was induced by randomly giving an empty water bottle to rats during watering periods per day for 14 consecutive days. The behavioral, endocrinological and immunological consequences were investigated. The data showed that the emotional stress activated both the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, leading to the increased blood levels of corticosterone and catecholamine. It also elicited attacking and exploring behavior, suppressed the immune function of the rats, including leukocyte counts, weight of the spleen, and the level of specific anti-ovalbumin IgG antibody production. Presenting no water and no empty bottle to rats only evoked the exploring behavior, increased the corticosterone level and decreased the leukocyte counts. These findings demonstrate a role of psychological factors on behavioral, endocrinological and immunological functioning. The animal model described in the present study may serve as an analogue mimicking emotional stress experienced in humans (e.g. anger and/or anxiety), and may be useful for further studying the complex relationships among emotional stress, behavior, and immune function.**

Keywords: emotional stress model, attacking, corticosterone, catecholamine, immune function.

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Stress has long been known to affect general health and increase susceptibility to diseases such as infectious disease, cancer, coronal heart disease, and autoimmune disease[1−4]. To understand the behavioral and physiologic processes of stress, research has been done mainly on animal subjects. Many animal models have been developed in an effort to reproduce different physiological effects of stress, including immunological effects[5,6]. These models may be divided into three main categories with respect to stressors used, namely, physical, social, and psychological stressors. Although none of these animal models contain pure aspects of one of these categories, most models employed, such as restraint, loud noise, and electric shock, are likely to be physical stressors producing physiological stress components[7]. As to psychological stressors, aside from social stressors such as isolation and maternal separation, which may be thought of as a special class of psychological stressors[8], conditioned aversive stimuli, controllable versus uncontrollable shocks have been commonly used[9]. These models involve learned responses originally linked to physiological stressors, and seldom involve behavioral or emotional measures.

Recent psychoneuroimmunological studies have primarily considered the contribution of psychological stress and its related behavior on immunological functioning. The consequences of the emotional stress may be related to the mechanisms of increased susceptibility to disease in humans[1]. Hence, the present study tries to describe a new model of emotional stress and examine its behavioral and physiological consequences. In this study, the emotional stress was induced by randomly presenting an empty water bottle (EB) to animals which were trained to drink water at scheduled time. Ovalbumin (OVA), a novel antigen, was used to induce the humoral immune response. The effect of emotional stress on behavioral, neuroendocrinological and immunological responses was investigated.

1 Materials and methods

(ⅰ) Subjects. Fifty-nine adult male Wistar rats about 60-d-old and weighing 220—250 g served as subjects. All animals were housed individually under controlled temperature conditions (20±2℃) with a 12-h light/dark cycle (lights on 07:00—19:00). Rats were given food and water *ad libitum* except during testing. Animals were acclimated to the laboratory environment for one week and gently handled each day, 3 min/d, to minimize the stress effects of handling. After the acclimation period, animals were trained to drink water between 9:00 and 9:10 am and between 9:00 and 9:10 pm by allowing them access to water bottles only during these time periods for one week. The 10-min drinking duration chosen was based on the observation that water-deprived rats normally spent 5—8 min continuously drinking when were allowed to access to water. After training, all rats were injected with 100 μg ovalbumin (OVA) (Sigma, USA) antigen i.p. in PBS emulsified in an equal volume of Freund's complete adjuvant (Sigma, USA).

(ⅱ) Experimental procedure. From the day of immunization, animals were divided into three groups: Group EB (empty water bottle), Group NB (no water bottle), and Group C. Animals in the EB group (n = 22) were randomly given empty water bottles during one of the two watering periods each day for 14 d. Animals in the NB

group (n = 18) were given neither water bottle nor water during the same watering periods as the rats in the EB group. Group C (n = 19) served as the controls. They were allowed free access to water during all watering periods. Rats in the EB and C groups were housed in one room and rats in the NB group were housed in another room to avoid the possible psychological influence of seeing other animals drinking or exhibiting stress behavior. All animals were killed by decapitation after completing the fourteenth test session. Trunk blood was collected and centrifuged for serum and plasma. Spleens were removed and weighed.

(iii) Analysis of behavior. Behaviors of all rats were observed during each of the 10-min experimental sessions through one-way screen windows. The behaviors observed included attacking (biting or attacking the empty water bottle and cage shed), exploring (rearing and horizontal motor activity, visiting the place of the bottle), and grooming (self-groom, wash and scratch). Each behavior pattern was recorded 4 times during experimental session. If the animal exhibited a particular pattern of behavior, the score of that behavior was recorded as 1, otherwise as 0. Thus the evaluation of each behavior item was finally added up as 0, 1, 2, 3, 4 scores respectively. Scores across two observers, one of them blind to the experimental conditions, were averaged. A mean of the scores for the last 3 d of 14-d test period was used for statistical analysis.

(iv) Determination of corticosterone and catecholamine levels. Corticosterone levels in serum were measured using modified radioimmunoassay (RIA)[10], and catecholamine (epinephrine, norepinephrin) levels in plasma were determined using high performance liquid chromatograpy (HPLC) with electrochemical detection[11].

(v) Determination of anti-OVA antibody production. Serum levels of IgG antibodies to OVA were determined by enzyme linked immunosorbent assay (ELISA). Microtiter, 96 well, flat-bottomed plates were coated with OVA (100 μL/well, 1 mg/mL OVA) and left overnight at 4℃. In the following day they were washed twice with Tween-phosphate buffered saline (0.05% Tween 20, pH 7.4) and once with double distilled water. The plates were then blocked for 1 h at 37℃ with 10 mg/mL BSA in PBS (Tween 20 0.005 μL/mL), 100 μL/well and then washed again. 1 : 100 dilutions of serum were prepared using BSA containing Tween 20 at a concentration of 0.005 μL /mL. Each plate was used for samples of different groups, three wells per sample, 100 μL /well. The plates were then incubated for 1 h at 37℃. All wells were washed again and 50 μL/well of goat anti-rat IgG antibody (Sigma), diluted 1 : 5000, were added. The plates were then incubated for 1 h at 37℃ and washed once again. Finally, 100 μL of enzyme substrate were added to each of the wells. Color was allowed to develop for 10 min and the reaction was stopped by the addition of 50 μL of 2N H_2SO_4. The optical density (OD) of each well was read on an EIA plate reader at a wavelength of 490 nm [12].

(vi) Determination of spleen weight and peripheral leukocyte counts. Trunk blood was collected in a heparinised tube. The number of leukocyte was counted in a JXJ-6 haemocytometer. Spleens were measured and expressed by the spleen indexes (spleen weight (mg)/body weight (g)).

(vii) Statistical analysis. The data were analyzed by a one-way analysis of variance (ANOVA) followed by the LSD of post hoc tests. In all tests, $P < 0.05$ was taken as the level of significance.

2 Results

(i) Effect of emotional stress on behavior. The mean scores of behavioral reactions to emotional stress for the last 3 d of 14-d test period are shown in Table 1.

Table 1 The mean scores of behavioral reactions during emotional stress for rats of different groups

Group	Attacking behavior	Exploring behavior	Grooming
C	0.0 ± 0.0	0.2 ± 0.042	3 ± 0.10
NB	0.0 ± 0.0	2.7 ± 0.48**	0.4 ± 0.052**
EB	2.8 ± 0.42**	2.6 ± 0.52**	0.2 ± 0.042**

C, control; NB, no water bottle; EB, empty water bottle. **P < 0.001, compared to rats of C and NB for attacking; compared to C for exploring; compared to C for grooming.

Attacking behavior was observed significantly in rats of the EB group when exposed to the stress procedure, but not in rats of NB and C groups, suggesting that rats in the EB group were extremely behaviorally stressed. Rats of both groups EB and NB expressed significant exploring behavior compared to rats of the Control group (NB vs. C, P<0.001; EB vs. C, $P < 0.001$), but there was no difference between group EB and group NB. These results suggest that rats in group NB were also stressed to some degree. For the rats of the control group, grooming was significant as compared with the rats of groups EB and NB (NB vs. C, P<0.001; EB vs. C, $P < 0.0001$).

(ii) Effect of emotional stress on the immune response. Changes in the values of immunological variables of different groups are presented in Table 2. Leukocyte counts of groups NB and EB were reduced significantly when compared to the C group (NB vs. C, $P < 0.05$; EB vs. C, $P < 0.05$), leukocyte counts were reduced by 28% in group NB, and 23% in group EB.

The spleen index (spleen weight (mg)/body weight

Table 2 Effect of emotional stress on anti-OVA antibody production, counts of leukocyte, and relative spleen weight

Group	Anti-OVA antibody /OD	Leukocyte /cells • μL^{-1}	Spleen weight /mg • g^{-1}
C	1.11± 0.21	870 ± 4463	1.97 ± 0.23
NB	1.21± 0.18	6226 ± 2380*	1.82 ± 0.23
EB	0.91± 0.21*	6688 ± 2082*	1.62 ± 0.17**

Data expressed as M±SD. *$P < 0.05$, compared to NB for anti-OVA antibody, compared to C for leukocyte; **$P < 0.01$ compared to C for spleen weight.

(g)) of group EB was lower significantly than that of groups NB and C (EB vs. NB $P < 0.05$; EB vs. C, $P < 0.01$). No significant difference in spleen index was found between group NB and group C.

The OD level of anti-OVA antibody at the 1 : 100 dilution of serum for different groups is presented in Table 2. In comparison with that of group NB, the level of anti OVA antibody production in rats of group EB decreased significantly ($P<0.05$). There was no significant difference in the level of anti-OVA antibody production between group NB and group C or between group EB and group C.

(iii) Effect of emotional stress on epinephrine, norepinephrine and corticosterone. As shown in Table 3, the epinephrine level of rats in group EB increased significantly as compared to that in group C ($P < 0.01$) and there was no difference between groups NB and C. The norepinephrine level of rats in group EB increased significantly as compared to that of both group NB and group C (EB vs. NB or EB vs. C, $P < 0.05$), there was no difference between groups NB and C. Corticosterone levels of rats in both groups EB and NB increased significantly as compared to that in the control group (EB vs. C, $P < 0.01$, NB vs. C, $P < 0.05$).

Table 3 Mean plasma levels (M±SD) of epinephrine, norepinephrine, and corticosterone for rats of 3 groups after 14-d experimental sessions

Group	Epinephrine /ng • mL^{-1}	Norepinephrine /ng • mL^{-1}	Corticosterone /ng • mL^{-1}
C	3.84 ± 1.28	1.11 ± 0.43	28.77 ± 23.60
NB	4.38 ± 1.55	1.14 ± 0.52	62.30 ± 28.10*
EB	5.59 ± 1.85**	1.71 ± 0.64*	79.93 ± 20.87**

*$P < 0.05$, **$P < 0.01$, compared with C for epinepherine; compared to C and NB for norepinephrine; compared with C for corticosterone.

3 Discussion

The aim of this work was to describe a new animal model of emotional stress, which was induced by randomly giving an empty water bottle to rats during watering periods per day for 14 consecutive days. The data, with the previous reports [13,14], showed that the emotional stress increased blood levels of corticosterone and catecholamine, suppressed the immune function of the rats, including leukocyte counts, weight of the spleen, and the level of specific anti-ovalbumin IgG antibody production. It also elicited attacking and exploring behavior. Pang et al.[15] further found that the learning and memory capabilities of the offspring could be impaired when pregnant rats were subjected to such emotional stress condition.

While the repeated results demonstrate that such a model of emotional stress was stable and applicable, several points should be addressed.

Firstly, rats, trained to drink water at set times, were also somewhat stressed when they did not get water at the watering time because the blood level of corticosterone, usually as an index of stress, increased significantly in group NB in comparison with that of control groups. They also exhibited exploring behavior. However, such NB stress situation did not affect the antibody level and the spleen index, and it also did not activate the sympathetic systems. With differential effects found for EB versus NB, the pure psychological contribution to the behavioral, neuroendocrine and immunological functions can be dissected through a comparison of the two groups. Such comparison suggests that the sympathetic nervous system is only activated by the psychological manipulation, namely by empty bottle stimulation. It also suggests that the suppressed leukocyte counts in both groups EB and NB are related to the increased corticosterone levels, whereas the suppressed humoral immune response in group EB is related to the increased levels of catecholamines, particularly norepinephrine. Such a notion is reinforced by a correlation study in which an inverse relationship between antibody formation and norepinephrine level was found[13].

It has been demonstrated that stress activates the SNS and the HPA axes leading to the release of catecholamines from sympathetic nerve terminals and from the adrenal medulla and glucocorticoid (corticosterone for rats) release from the adrenal cortex. Both catecholamines and glucocorticoids have been shown to affect immune responses[16,17]. Our data suggest that catecholamine and corticosterone may have a differential effect on humoral and cell-mediated immune function. The sympathetic nervous system may be more involved in humoral immunomodulation of emotional stress. The innervations of immunological organs by the sympathetic nervous system and the presence of adrenergic receptors on various immunological cells such as T and B cells provide a structural basis for mediating mechanisms between the CNS and the immune function[18]. But how the SNS is involved in the humoral immunosuppression caused by emotional stress requires further investigation.

Secondly, the effect of emotional stress on humoral immune function depends on the consecutive days of empty bottle presentation. In our previous study[14], it was found that 3-d empty bottle presentation had somewhat but not significant effect on anti-OVA antibody production while the 14-d empty bottle presentation significantly suppressed the antibody production. These results suggest

that there may exert an accumulative effect of emotional stress on body reaction in terms of humoral immunity.

The other point should be noted is that there was a significant difference in antibody production between the EB and NB rats, but not between groups NB and C. These data may suggest that strong stress suppresses antibody production, whereas mild stress may somewhat enhance antibody production. Therefore the difference in antibody production between EB and NB would be greater than that between groups EB and C.

Finally, regarding the models of emotional stress, most studies on animals have commonly evaluated emotionality under experimental conditions where some fear-like emotion is expected to be experienced. For example, Blanchard et al.[19] described a model of exposing rats to cat as emotional stress and Croiset et al.[20] adapted one-trial-learning passive avoidance test linked to electric foot-shock as emotional stress. In humans, emotions have been classified into different categories, such as fear, anger, anxiety, sadness, shame, etc.[21]. The emotion experienced by animals in the present study seems to be more related to anger and/or anxiety.

In summery, the two types of stressor (EB vs. NB) described in this experiment elicited different behavioral, neuroendocrinological and immunological reactions. They model different degrees of stress as well as different types of emotional reaction and mood disorder, which may serve to mimic very specific human stress situations (e.g. anger and/or anxiety).

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