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ORIGINAL ARTICLE

Estradiol Level of Male Rat is Correlated with Depression and Anxiety after Traumatic Stress

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Post-traumatic stress disorder (PTSD) is a complex syndrome that is defined by individual exposed to intense, life-threatening trauma thereby leading to physical and psychological abnormalities. They further develop additional symptoms including persistent anxiety, exaggerated startle, cognitive impairments, and diminished extinction of fear. Most of the previous studies focused on brain and neurophysiology, but PTSD affects the whole body. Therefore, we wanted to concentrate more on the effects on sex hormones using rats. Testosterone and estradiol are major sex hormones in male and female which are well known to participate in not only reproduction but also brain function and behaviors. Two behavioral tests, open field test and tail suspension test, were used to determine whether animals have PTSD-like symptoms. At the same time, serum samples were collected for sex hormone analysis. We investigated the influences of traumatic stress and then tried to find out the correlations of behaviors and hormones. In results, 22% of rats were affected by stress based on the behavioral tests and grouped as PTSD-like. These rats showed enhanced anxiety and depression behaviors. In serum samples from the PTSD-like group, only estradiol levels in male after stress are significant higher than the control or stressed but no symptom groups. Testosterone level showed no difference after stress both in male and female. We also observed that only estradiol level in male correlates with PTSD-like behaviors. It indicates that estradiol levels in male just after traumatic stress might be an indicator for PTSD-like symptoms.

Key words: Posttraumatic stress disorder, sex-hormone, estradiol, depress, anxiety

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a kind of mental disorder that occurs in people, who have experienced a scary or dangerous incident, such as war, traffic accident, sexual assault, or other survival stress. The population of PTSD is increasing because of terrorist attack and natural disaster, and arouses the concern in recent years. It shows complex and multiple symptoms in PTSD patients including re-experiencing the trauma, persistent anxiety, exaggerated startle, cognitive impairments, and depression.[1] PTSD is usually diagnosed by some physical examinations and psychological evaluations. It is well defined that PTSD patient is at a higher risk for suicide and intentional self-harm.[2] Furthermore, there are many evidences show that a man with PTSD also has some abnormal in other systems besides mental.

Most studies of PTSD focus on the brain because it is the major area implicated in stress response includes the amygdala, hippocampus, and prefrontal cortex. Studies indicated that traumatic stress lasting impact the brain in structure and function of multiple areas.[3] Besides the neurobiological abnormalities, cardiovascular disease is associated with PDST.[4] Furthermore, other symptoms of PTSD in the whole body were shown such as physical pain,[5] immune system dysfunction,[6] and hormone imbalance.[7] Patients with PTSD showed increased cortisol and norepinephrine responses.[8] Besides adrenal hormone, sex hormones play important roles in mental functions but seldom been a link to stress response.

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It is well known that sex hormones, such as estrogen, progesterone, and testosterone, have extended the pivotal actions outside of the reproductive tract. Estrogen actions occur in brain regions involved in learning and memory, emotion, motivation, and cognition.[9] Ovarian hormones exhibit a profound effect on neurotrophins such as brain-derived neurotrophic factor to regulate neurotransmitter system and affect the neuronal survival.[10] Besides neuroprotection function, estradiol affects the neurochemical systems involved in emotional and cognitive control such as dopaminergic and serotonergic systems.[9] Estradiol supplement to ovariectomized (OVX) or aged animals have anxiety- and depression-reducing effects,[11] and similar results were found in the human study.[9] On the other hand, testosterone replacement in older men demonstrates improvement in spatial cognition, verbal memory, and working memory.[12,13] Several evidences also suggest that testosterone might be helpful in the treatment of depressive disorder in men.[14]

Accordingly, estradiol and testosterone are important to physically function of the brain and seems effective for certain mental disorder and depression. However, there are a few studies that mention about the sex hormone level in PTSD. Therefore, we would like to analyze the relationship between depression and anxiety behaviors with sex hormone by an animal model which is easy to collect data before and after traumatic stress. Anxiety and depression are two major symptoms while exposure to trauma and stress, thus behavior analysis of anxiety and depression was important for PTSD diagnosis. There are several well-defined animal models for depression, such as forced swim test (FST) and tail suspension test (TST).[15] The animals will be put into water or hung upside-down and will struggle generally. However, depression animals will learn helplessness and give up. On the other hand, the anxiety of rodents could be measured by elevated plus-maze or open field test (OFT). Although anxiety is a normal adaptive response to danger, PTSD is a disease of anxiety disorder. Our purpose is to evaluate the sex hormone changes by traumatic stress, and verify whether the hormone changes correlated with depression and anxiety behaviors.

MATERIALS AND METHODS

Animals

Seventy adult Sprague-Dawley rats weighing between 250 and 300 g were supplied by BioLASCO Taiwan Co., Ltd. (36 male/34 female; 10 male/10 female for control experiment without any traumatic stress). The animals were housed in groups of three at a constant cage temperature (22°C ± 1°C) and humidity (40%–70%). The animals were allowed to adapt for 2 weeks to the novel environment before any experiment was performed. First time of behavior test and blood collection was performed at 3rd week after arrived. They were kept under regular light-dark conditions (light on at 7:00 am and off at 19:00 pm) with food and water available *ad libitum* except during behavioral testing and traumatic stress. Rats were randomly assigned to stressed or control groups.

Traumatic stress procedure

Restraint stress

The traumatic stress was performed after 4 weeks after arrived. For restraint like the previous study,[16] an individual rat was put in a 50 ml sterile tube for 180 min. The tube was made of plastic that offered the same lightning and temperature as in the large home cage but without water and food. The plastic tube measured 11.5 cm in length and 3 cm in diameter. Rats would be restricted totally and could not move back and forth neither could turn around while in the tube.

Predatory exposure

Rats were randomly assigned to exposed or control groups. Subjects of the exposed group were confronted individually with a cat small and sound.[17] Real cat exposure to laboratory animals may cause infection risk, only odor from cat litter and sound from the video were used in the experiments. The rats were not able to escape from this compartment or from the cat, though they were not physically injured. After 180 min of exposure to the cat, the rat was put back in its cage first. All the traumatic stresses execute in the present study including restraint stress at morning and predatory exposure at afternoon in the same day around 1 month after arrived.

Anxiety/depression test

Open field test

The animal was placed in the center of the activity field arena similar to the previous study,[18] which is a transparent plexi cage (width [W] × depth [D] × height [H]; 48 cm × 48 cm × 40 cm) equipped with two photobeam sensor rings to register horizontal and vertical activity. Testing lasted for 15 min. The entire rat walking distance and the entrances into the center zone (W × D; 24 cm × 24 cm) were to be tracked and counted by video and analyzed through software (TopScan Lite 2.0 (Clever Sys., Reston, VA, USA)).

Tail suspension test

The rat was suspended by their tail to a hook in a test chamber using adhesive tape. The total duration of immobility was measured over a period of 5 min using the video track system (Viewpoint, France) just like the previous study.[19] Only the immobility was continued for 7 s was counted as 1 frequency of immobility. A rat that curled up toward their tail or that fell off during testing was excluded from the analysis.

Hormone detection (ELISA) of estradiol and testosterone

Blood samples (500 ul) were collected through a tail vein of rats, and the samples were kept in 1.5-ml Eppendorf tubes on ice. Serum was separated by centrifugation at 4°C and stored at − 20°C. Serum hormone levels were determined through an ELISA-based direct sandwich technique. Two of ELISA kits were used to determine the levels of estradiol (#ES180S-1000; Calbiotech) and testosterone (#582701; Cayman). A standard curve was generated through the reference standard dissolved in the medium. All samples were analyzed according to the company instructions. A microplate reader (Luminescence Scanner, Thermo Inc., Waltham, MA, USA.) was used to detect the signals at wavelengths of 450/415 nm. The plasma hormone concentration was calculated based on the standard curve and presented in nanograms per milliliter.

Statistical analysis

Data were expressed as mean ± standard error of mean and analyzed with one-way ANOVA followed by Newman–Keuls comparisons. Values of $P < 0.05$ were considered statistically significant. In the correlation analysis, control group was excluded, and only animals expose to traumatic stress were analyzed. The hormone data from animals at first-time detection after traumatic stress were used, and data of behavior test were also calculated form the results after traumatic stress. The linear diagram and values of R^2 were shown using excel automatically.

RESULTS

Experimental chart and body weight of rats

The animals were allowed to adapt for 2 weeks to the novel environment before any experiment was performed. First time of behavior test and blood collection was performed at the third week after arrival. The traumatic stress which combined predatory exposure and restraint stress was performed at 30 days after arrived. After stress, the blood samples and

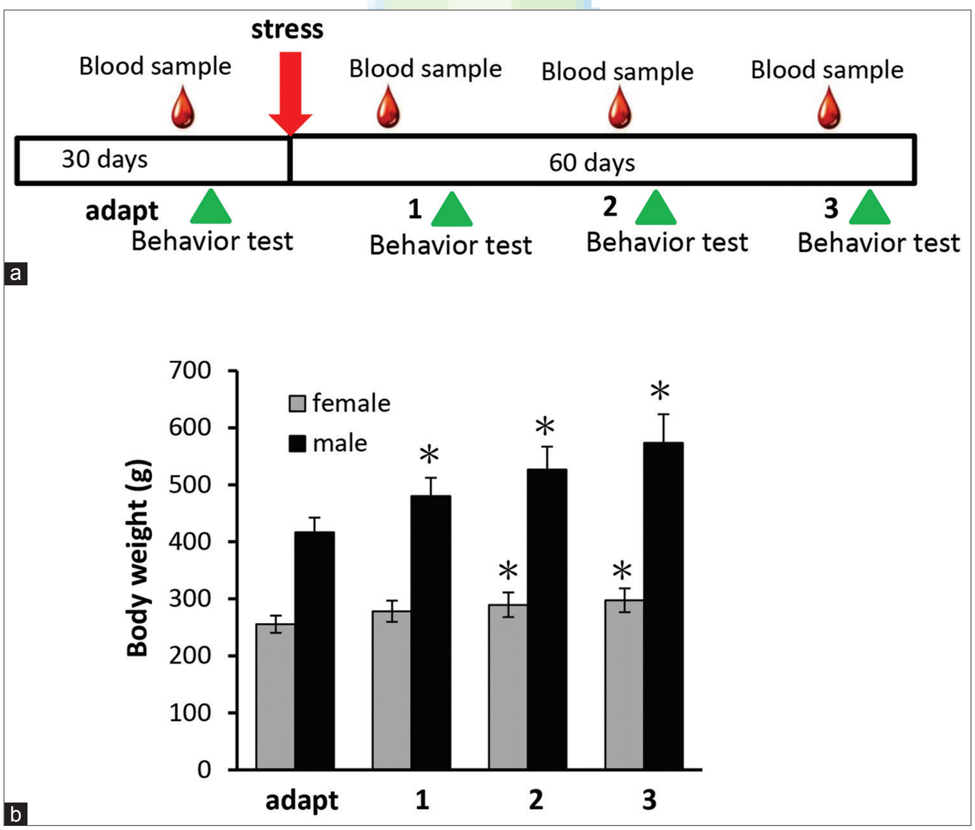

Figure 1: The experimental chart and body weight of rats (a) red arrow sign indicates the day of traumatic stress. There is one blood collection (indicated by teardrop). One behavior test (indicated by a triangle) before stress (in adaption) and three times after stress. (b) Body weight of rats during the experimental period. The gray box represents all female rats ($n = 34$), and black box represents male rats ($n = 36$). Numbers (1, 2, and 3) under panel indicate the data from first, second, and third measurement after stress, respectively. Body weights are increased compared to the first measurement in adapt. (*$P < 0.05$)

behavior analysis were collected for three times. There are four times of both blood detections and behaviors tests of total experiments [Figure 1a]. The body weights at different time were measured just before blood collection every time. Results showed that female rats were smaller than male. The body weight of female rats at the beginning was 250 ± 15 g, and 300 g ± 21 g at the end; bodyweight of male rats was from 417 ± 25 g to 573 ± 50 g. Both male and female rats gain weight gradually as time period; however, there was no significant difference after traumatic stress [Figure 1b].

The frequency of immobility in tail suspension test

TST was a model used to measure the depression of animals.[19] Struggled rat would turn over and try to escape an aversive stimulus [Figure 2a left]; however, immobile rat would stop trying and give up [Figure 2a right]. If the immobility was last for 7 s, we counted as 1 frequency. In the results, the frequency of immobility was shown; the values were between 2 and 26, and the variations were high. We found that female rats seem have lower immobility rate than male but not significantly. Furthermore, because of the variation, there was no significant difference either compares adapt or control group to the stressed animals [Figure 2b]. We next analyzed the results of all stressed animals, and found some rats showed obviously changes in behavior test after stress. There are five female and six male rats revealed significantly difference in both TST and OFT before and after stress. We defined these rats as the PTSD-like group because they were more depression and anxiety just after traumatic stress. Except PTSD-like rats, other animals were grouped to SN, which means stressed but no symptoms (SNs) (female n = 19; male n = 20). While separate the stressed animals into two groups, we could find that PTSD-like rats showed higher immobility rate (2–3 fold higher) in both male and female compare to SN groups and control (female n = 10; male n = 10) [Figure 2c].

The frequency of entrance into center area in open field test

OFT is a well-known model for animal behavior test of anxiety.[18] Rodents prefer to stay around the close area rather

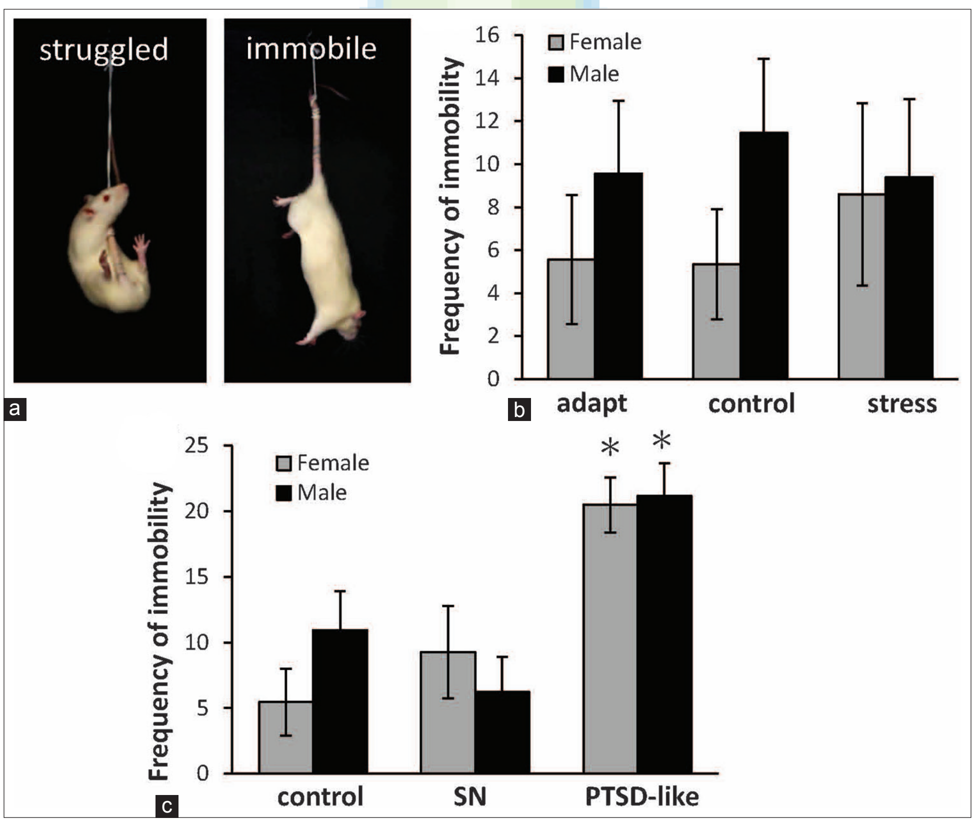

Figure 2: Effects of traumatic stress were evaluated by tail suspension test. (a) Pictures reveal different behavior in tail suspension test. (b) In both male and female, the frequencies of immobility are no difference among adapt, control, and stressed group. (c) We group the rats showed obviously changes in behavior tests after stress as the post-traumatic stress disorder-like rats. Other stressed rats without symptoms called stressed but no symptom rats. Control rat's mean without stress expose. Therefore, the post-traumatic stress disorder-like group shows high frequencies of immobility compare to others (*P < 0.05)

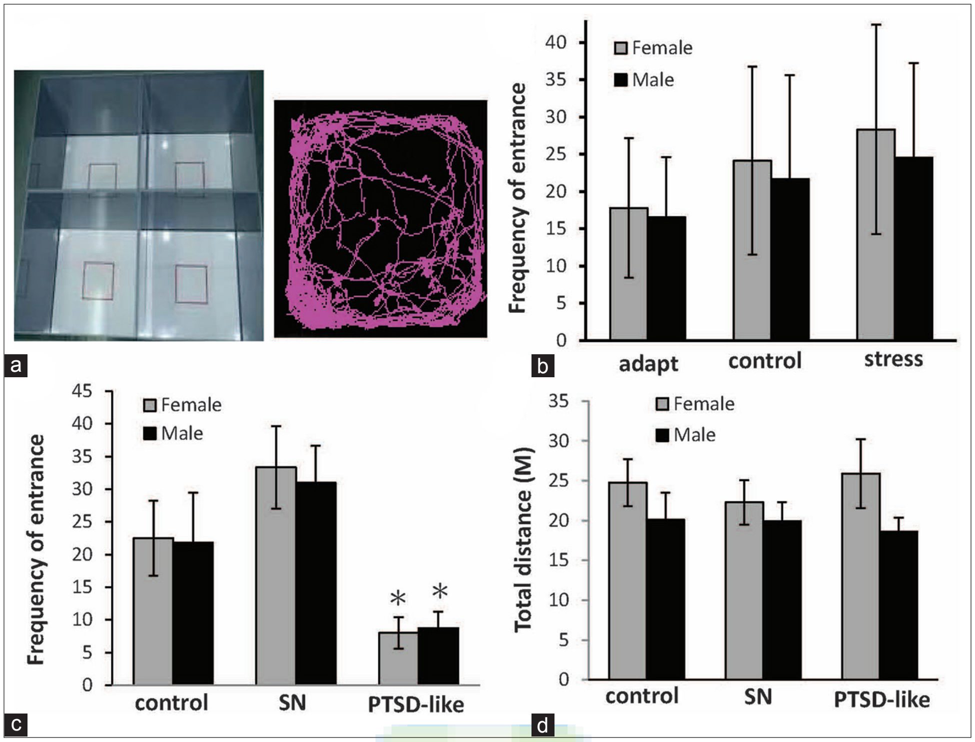

Figure 3: Assess the influence of traumatic stress by an open field test. (a) The equipment of open field test was shown. The track of rats was recorded by video and shown as the pink lines, and the frequency of entrance into the center area were counted by software. (b) The entrance into the center area is no difference among adapt, control, and stressed group in female (gray) and male (black). (c) post-traumatic stress disorder-like rats reveal a significant decrease in open field test compare to control and stressed but no symptom groups (*$P < 0.05$). (d) Total distances of open field test are similar among all groups represent the moving activity of all animals are not affected

than open place. If the animal would like to explore the open area means it has less anxiety. The equipment of OFT was shown in Figure 3a, and the red squares revealed the center area which was the open place for animals. The track of rats was recorded by video and shown as the pink lines [Figure 3a], and the frequency of entrance into center area was counted by the software. The results [Figure 3b] showed that both male and female rats exposed to traumatic stress have similar entrance frequency (around 20–30 times) to unstressed control and all of the animals in adaption (before stress). However, similar to TST test, the PTSD-like animals were been chosen by their obviously decrease in the entrance frequency (only 8 times) [Figure 3c]. The group of SNs showed no significant difference compare to control. To realize the move activity, total distance in OFT was also collected. According to the results, they showed equal ability of movement in all groups [Figure 3d].

Sex hormone analysis of rats in different groups

After behavior tests, we investigate the sex hormone level form serum collected at different time points. At first, we found that female rats had few testosterone [Figure 4a] and male rat also showed low amount of estradiol [Figure 4b] as expected, and there were no difference among stressed animals, unstressed control and those in adaption no matter in testosterone [Figure 4a] or estradiol [Figure 4b]. Then hormone level was showed in different groups at different time points. In testosterone level, female revealed low concentrations (around 30–40 pg/ml) and no significant difference in three groups at different time points [Figure 4c]. Testosterone in male rat is high (around 2000 pg/ml), but there was still no difference in three groups at different time points [Figure 4e]. On the other hand, estradiol level was high in female (around 250 pg/ml) [Figure 4d] and low in male (30–50 pg/ml) [Figure 4f]. Although the estradiol level remained no significant change in female at different times in all groups [Figure 4d], it showed dramatical increase (8–9 fold higher) in PTSD-like group of male rat just after traumatic stress [Figure 4f]. According to the results, only estradiol level in male rats those had PTSD-like symptoms revealed significant changes after stress.

The relationship between behavior test and sex hormone

We were surprised to find that the PTSD-like male rat had enhanced estradiol level after stress. To verify the relationship

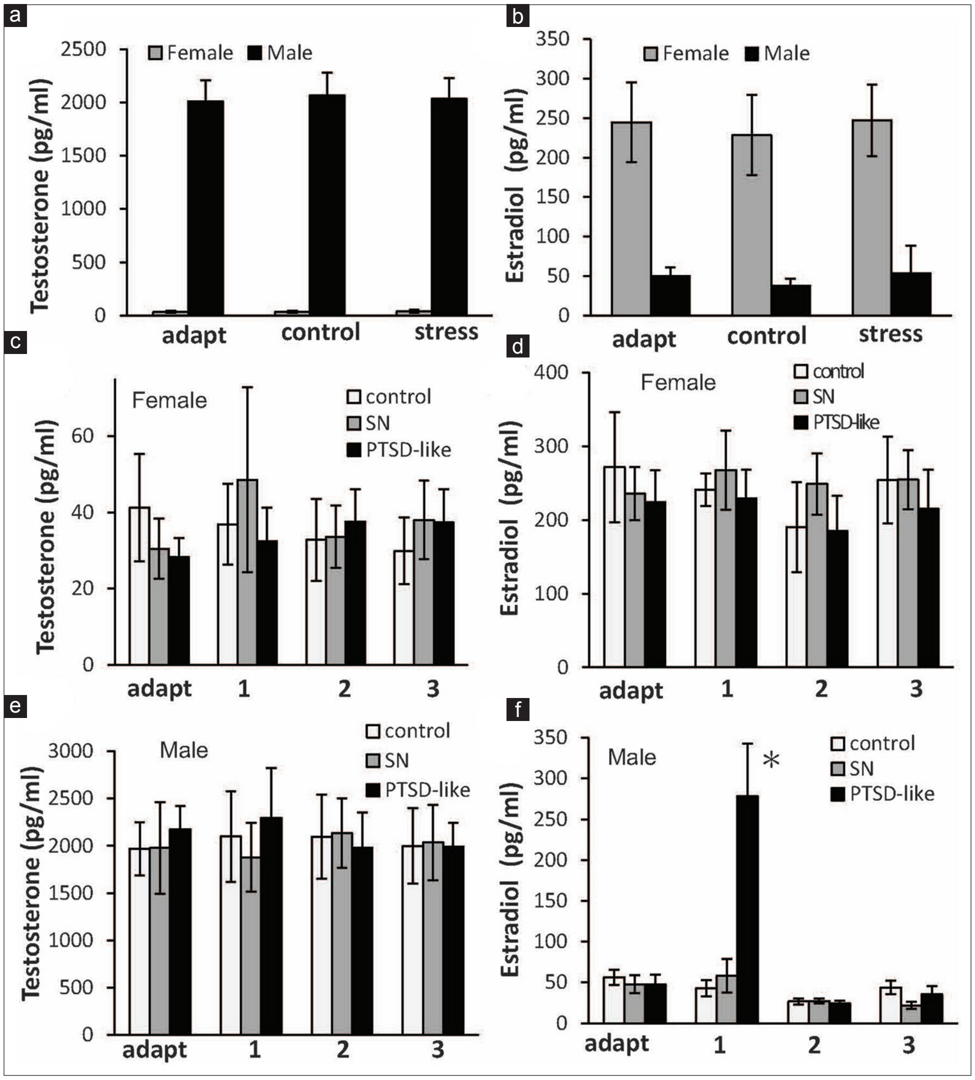

Figure 4: Analyze the changes of sex hormone in different groups before and after stress. Testosterone (a) and estradiol (b) showed no difference among adapt, control and stressed rats. Comparing testosterone (c) and estradiol (d) at a different time in female showed no difference. Adapt means before stress, and numbers (1, 2, and 3) indicate the data from first, second, and third blood sample after stress, respectively. Comparing testosterone (e) and estradiol level (f) in the male at the different time. Only testosterone of post-traumatic stress disorder-like male elevated at first blood test (*$P < 0.05$)

between the behavior and hormone, the correlation analyses were done. The hormone data from male rat at the first-time detection after traumatic stress were used, and data of behavior test were also calculated form the results after traumatic stress. We found that the immobility of TST was positively correlated ($R^2 = 0.44$) with estradiol level in all stressed male [Figure 5a], and the animals had higher estradiol also showed reduced in entrance into center area of OFT ($R^2 = 0.52$) [Figure 5c]. However, the entrance into the center area of OFT was low correlation with testosterone ($R^2 = 0.2$) [Figure 5d], and the immobility of TST had no correlation with testosterone ($R^2 = 0.12$) [Figure 5b]. The results implied that male rats with depression and anxiety behavior probably had higher estradiol in their body just after the traumatic stress.

DISCUSSION

According to the results, we found that stressed male rats which were more depression and anxiety revealed higher estradiol level in serum, but testosterone level did not showed any correlation with behavior. Sex hormones in female rats after traumatic stress did not correlate with the results from behavior tests either. Thus, only male could use the estradiol level as the indicator of anxiety- or depression-like behavior after stress. The gender differences may due to the difference in basal hormone level. Because of low background of estradiol in male (around 40 pg/ml), the stimulation effects of traumatic stress became obvious (up to 5–7 folds). While average concentration of estradiol is 260 pg/ml in control female rat, further activation was difficult to been shown, and

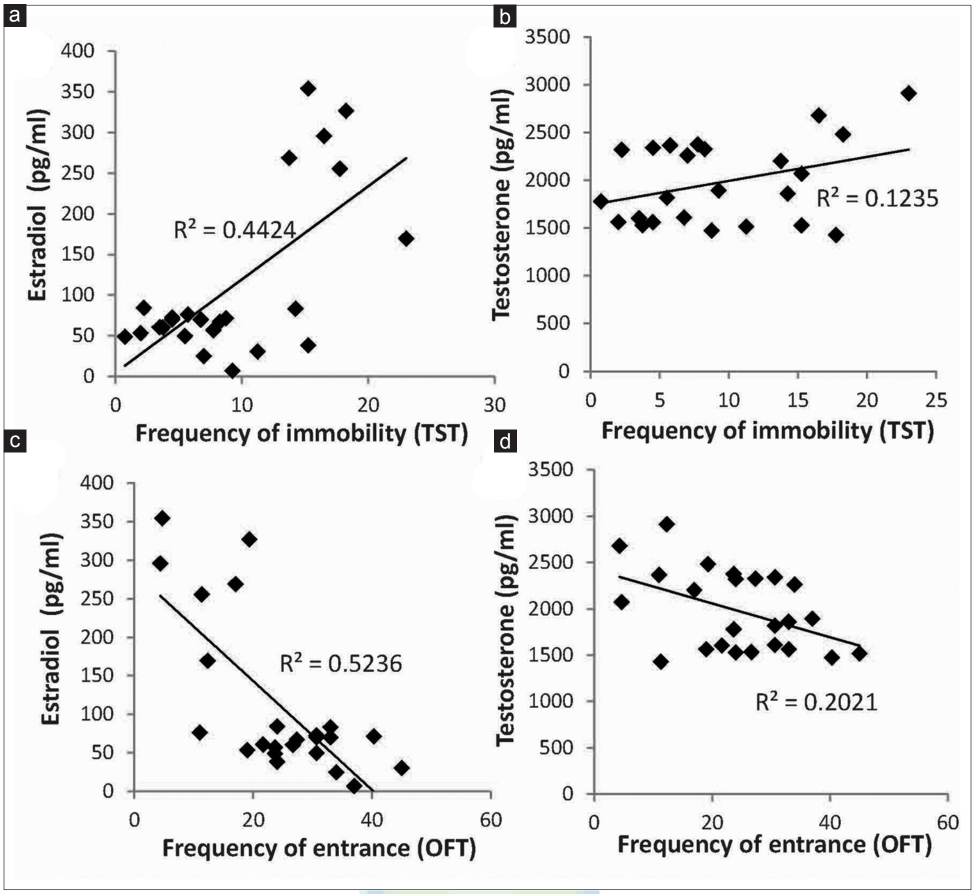

Figure 5: Correlations of sex hormones with behavior tests in male rats estradiol levels (a) positively correlate with tail suspension test, but testosterone (b) levels have no correlations with tail suspension test. In anxiety behavior test, estradiol levels (c) negatively correlate with open field test, and testosterone (d) levels remain no correlations with tail suspension test

the variation is also high in female because of subtle fluctuations across the estrus cycle. Therefore, female animals at the same cycle stage or after OVX may be better for further study.

Furthermore, not only estradiol changes in estrus cycle but also the mood disturbance. It is well known that a subgroup of women suffers from a clinical level of premenstrual mood changes called premenstrual dysphoric disorder (PMDD).[9] Symptoms of PMDD include anxiety, irritability, and depressed mood that are somehow similar to PTSD. Another key fact has been mentioned that cycling estrogen level in female associated with the fear inhibition. Comparing the men, women with high estradiol levels, and women with low estradiol (LE) level, LE women showed significant reduced in the extinction of conditioned fear.[20] In the rat, estrogen also modulates sexually dimorphic of fear extinction.[21] These results implied a possible role of estradiol in the extinction of fear memories, and the major event of PTSD is an insufficiency in fear extinction. It is well defined that estradiol supplement reduces anxiety- and depression-like behavior in both mice and human. Thus, it is not surprising that estradiol correlated with anxiety and depression behavior. However, we found estradiol enhanced in the PTSD-like group but not the SN group. The possible explanation is that only damaged animals have some physiology mechanisms to increase the production of fear extinction related hormones, such as estradiol. Another subgroup of stressed animal showed no difference in anxiety- and depression-like behavior might not suffer from the traumatic stress procedure, so the hormones level were not altered.

According to our data, only 22% (11/50) of rat revealed PTSD-like behaviors after stress. It is much lower than the previous study in a human which showed 40%–70% of individual exposed to intense, life-threatening trauma are at significant risk for developing PTSD.[22] We think the first possible reason is the species difference that rodents are tougher from survival stress than human. However, it is hard to study PTSD in human because the causes of PTSD are diverse and complicated and the symptoms are various in different periods after stress.[1] Furthermore, we separated the PTSD-like animals according to two kinds of behavior tests, TST and OFT. Only the animals with double symptoms (depression and anxiety) would be picked into the PTSD-like group. Accordingly, the number is fewer than depression or anxiety alone. Another reason is the intensity of traumatic stress. Compared to other study of PTSD model in rodents with an electric foot shock,[17] the intensity of traumatic stress using here seems weaker.

In the correlation study of sex hormone and behaviors, we first analyzed all animals and could not find any relationships between behavior tests and hormones (data not shown). Then after ruling out the unstressed control, we found the positive correlation between estradiol and TST results; and a negative correlation between estradiol and OFT results in male rats. Therefore, we think the estradiol level in male rat also altered by some other factors and it could be the biology indicator of anxiety or depression only at the period post-traumatic stress.

Actually, this is not the first study that has discussed about the relationship between behavior and sex hormones. It is well known that testosterone levels have correlated with aggression.[23] Insufficient of testosterone may contribute to depression,[24] confusion, irritability, and fatigue.[25] Furthermore, estrogen has a positive influence on cognitive function.[26] Most importantly, estrogen plays critical roles in the regulation of neurotransmitters,[9] neuroprotection,[27] and fear extinction.[20,21] However, the sex hormone functions in PTSD development are poorly understood. Our study is a novel finding that estradiol concentrations in man liked to stress-induced depression and anxiety. It implies a possible way to use the estradiol level as the indicator for PTSD diagnosis.

CONCLUSION

In male rat experience to traumatic stress, their sex hormone, estradiol but not testosterone was enhanced and correlated with depression and anxiety behaviors. In contrast, some female rats also revealed PTSD-like behaviors but without connections with sex hormone levels. These findings are novel and may provide a possible biology indicator for PTSD diagnosis.

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Conflicts of interest

There are no conflicts of interest.

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