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Evaluation of the Humoral Immune Response of Wistar Rats Submitted to Forced Swimming and Treated with Fluoxetine

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1. Introduction

The term stress was introduced into the biomedical field by Hans Selye (1936) in reference to a General Adaptation Syndrome which would consist of all non-specific systemic reactions that occur during an intense and chronic exposure to a stressor (e.g., pressure at work and poor diet). This syndrome would be different from the specific adaptive reactions (such as muscle hypertrophy caused by exercise performed on a regular basis) and immune responses (Selye, 1936).

A study evaluating occupational stress in nurses presented the most common symptoms involved: a feeling of fatigue, headache or muscle pain due to tension (neck and shoulders), decreased sexual interest, a feeling of discouragement in the morning, sleep difficulties, upset stomach or stomach pain, muscle tremors, feeling short of breath or shortness of breath, decreased appetite, tachycardia when under pressure, sweating and flushing (Stacciarini & Tróccoli, 2004). The main psychological symptoms present in people with stress are anxiety, tension, insomnia, alienation, interpersonal difficulties, self-doubt, excessive worry, inability to concentrate, difficulty relaxing, anger and emotional hypersensitivity (Lipp, 1994).

Stress has been considered one of the biggest causes of depression. After a situation of great stress, approximately 60% of individuals develop depression. Psychosocial problems (work pressure, job loss and debt) can also be preconditions for its emergence (Kendler et al. 1995; Post, 1992).

Major depression is a mood disorder whose prevalence throughout life, depending on the population, is estimated at between 0.9 to 18% and involves a significant risk of death (Waraich et al., 2004). It is estimated that men and women with depression are 20.9 and 27 times, respectively, more likely to commit suicide than those without depression (Briley & Lépine, 2011).

Multiple environmental factors have been associated with the etiology of depression. Adverse events during childhood and everyday stress are described as important factors for

the development of depression (Kessler, 1997). Children with a history of sexual abuse, living in troubled homes or who receive little attention from parents have a high risk of becoming depressed adults (Kessler, 1997). Stressful events such as the loss of a loved one, job loss, or partner separation are factors associated with the onset of depression (Kessler, 1997). Individual personality is also a predisposing factor to depression, as evidenced by the higher frequency of depression in people with a tendency to be sad when they experience a stressful event (Fava & Kendler, 2000). Gender is strongly associated with depression. Studies have shown that depression is on average twice as common in women as in men (Bromet et al., 2011). Interestingly, a decrease in the female/male proportion of depression has been observed in young adults (18 to 24 years), possibly due to greater gender equality in today's society (Seedat et al., 2009). Besides environmental factors, individual genetic characteristics also contribute to susceptibility to depression (Jabber et al., 2008).

In addition to the psychological changes associated with depression, immune system changes are often found in depressed individuals (Altenburg et al., 2002). Several studies have indicated that stress and depression involve the individual in a chronic process that results in host defense failure against microorganisms and a higher likelihood of developing certain cancers. These alterations are probably associated with profound changes in the functioning of the immune system of individuals suffering from depression (Reiche et al., 2004; Irwin et al., 2011). Epidemiological and experimental evidence shows that changes in the defense capability of the individual are related to decreased proliferative capacity of peripheral blood lymphocytes stimulated with mitogens in vitro (Schleifer et al., 1985; Schleifer et al., 1996), a decrease in the cytotoxic activity of natural killer cells (NK) (Schleifer et al., 1996; Calabrese et al., 1987; Nunes et al., 2002), the suppression of T-cell activity due to increased apoptosis and decreased cell proliferation in response to antigens (Szuster-Ciesielski et al., 2008; Schleifer et al., 1984). Moreover, imbalance in cytokine levels is often observed, such as increased levels of interleukin 2 (IL-2), interleukin 6 (IL-6) and interferon-alpha (IFN-α) (Seidel et al. 1995; Vismari et al., 2008). The results have been conflicting regarding humoral immune response and immunoglobulin levels in the blood. A significant increase in IgM levels in patients with depression was observed by Kronfol (1989) and Song et al. (1994), although other studies have been unable to detect significant changes in immunoglobulin levels in the peripheral blood of patients with depression (Bauer et al., 1995; Nunes et al., 2002). These changes in the immune system probably directly and/or indirectly compromise host immunity against microorganisms (Miller, 2010). On the other hand, the immune system changes observed in individuals with depression may not be caused by changes in the central nervous system of these individuals but instead may be directly related to the origin of such changes, including the development of a pro-inflammation state directly related to the onset of a depressive state, which is suggested by the hypothesis that macrophages act as a cause of depression (Miller, 2010). This hypothesis is related to an increased secretion of proinflammatory cytokines such as interleukin 1 (IL-1), IFN-α, and the resulting change in production of corticotrophin-releasing factor (CRF) and adenocorticotrophic hormone (ACTH) (Smith, 1991).

Importantly, animal models of stress and depression have shown immune system changes, including increased production of IL-1, the number of circulating neutrophils and lowered resistance to infection by bacteria. Mice that had been transgenically modified to exhibit a depressive type of behavior (catalepsy) and were inoculated with sheep red blood cells

(SRBC) had lower amounts of platelet-forming cells and antigen-specific T lymphocytes than their parents without this disorder. In rats with high levels of anxiety, lower concentrations of specific T lymphocytes were also found five days after inoculation with SRBC (Kubera et al., 1996; Pedersen & Hoffman-Goetz, 2000; Altenburg et al., 2002; Robles et al., 2005; Alperin et al., 2007; Loskutov et al., 2007; Miller, 2010).

Because this disorder severely compromises the functioning of individuals, several alternative treatments for depression have been proposed, including psychotherapy and pharmacotherapy, as well as a combination of both types. The use of antidepressant drugs for treating patients with depression began in the late 1950s. Since then, many drugs with potential antidepressants have been made available and significant advances have been made in understanding their possible mechanisms of action (Stahl, 1997). Only two classes of antidepressants were known until the 80's: tricyclic antidepressants and monoamine oxidase inhibitors. Both, although effective, were nonspecific and caused numerous side effects (Lichtman et al., 2009). Over the past 20 years, new classes of antidepressants have been discovered: selective serotonin reuptake inhibitors, selective serotonin/norepinephrine reuptake inhibitors, serotonin reuptake inhibitors and alpha-2 antagonists, serotonin reuptake stimulants, selective norepinephrine reuptake inhibitors, selective dopamine reuptake inhibitors and alpha-2 adrenoceptor antagonists (Bezchlibnyk-Butler & Jeffries, 1999). Serotonin reuptake inhibitors belong to this new generation of antidepressant drugs; fluoxetine is the most commonly prescribed drug for treating depression and anxiety because of its efficacy, safety and tolerability (Egeland et al., 2010).

Despite the current extensive use of antidepressant drugs, few studies have investigated the effects of antidepressant drugs on the immune system (Janssen et al., 2010). Experimental and clinical evidence suggests that changes in the immune system in patients with depression can be reversed by the use of antidepressant drugs (Leonard, 2001).

In animal models the use of fluoxetine has been associated with significant changes in immunity. Laudenslager & Clarke (2000) inoculated rhesus monkeys (*Macaca mulatta*) with tetanus toxoid and found increased levels of IgG anti-tetanus. When analyzing the effect of the antidepressant desipramine and fluoxetine, it was observed that animals treated with these antibodies showed higher plasma levels than those treated with saline.

Some studies with mice have showed the effects of fluoxetine on humoral immune response. Kubera et al. (2000) observed that continuous administration of fluoxetine in C57BL/6 mice for four weeks results in decreased IL-4 production and in increased IL-6 and IL-10 production. Genaro et al. (2000) found that fluoxetine has an inhibitory action on the proliferation of B lymphocytes induced by lipopolysaccharide (LPS) or anti-IgM. On the other hand, fluoxetine increases the proliferative action of B lymphocytes, being stimulated by suboptimal concentrations of anti-IgM. In an experimental model of depression in BALB/c, Edgar et al. (2002) observed a decrease in lymphoproliferative response induced by mitogens (phytohemagglutinin and concavalina A), an increase in the proliferative response of B lymphocytes to lipopolysaccharide (LPS) and that the chronic administration of fluoxetine reverses these immune changes.

The experimental investigation of depression in humans is largely ethically unfeasible. Thus, animal models of depression have been developed for this purpose, such as the

olfactory bulbectomy, learned helplessness, restraint stress and forced swimming (Willner, 1990). Forced swimming is a widely used model for preclinical evaluation of the possible effects of antidepressant drugs (Porsolt et al., 1977). Its widespread use is mainly due to its ease of implementation, the reliability of its results confirmed in various laboratories and its ability to detect the action of almost all classes of currently available antidepressants (Borsini & Meli, 1988).

In this study we evaluated the humoral immune response of rats chronically submitted to a model of stress/depression, i.e., forced swimming for twenty-five days and daily treatment with fluoxetine. Antibody production was assessed five days after the rats were inoculated with sheep red blood cells and, after the last day of forced swimming, the animals were euthanized and the adrenal glands, thymus and spleen were removed and weighed.

A growing number of people are diagnosed with stress and depression, for which antidepressant drugs are increasingly prescribed. Although many of their effects on individuals are known, there have been few studies reporting the effects of antidepressants on human and/or animal immune systems, especially regarding humoral immunity. Although experimental, this study has great social significance principally due to the large number of people vaccinated annually who are also undergoing regular treatment with antidepressants. The objective of this study was to evaluate the humoral immune response of Wistar rats submitted to forced swimming and treated with fluoxetine.

2. Methodology

2.1 Animals and experimental groups

A sample of 72 male Wistar rats with a body mass of about 300 grams was obtained from the Central Vivarium of the State University of Londrina's Center of Biological Sciences for use in the experiment.

The experiment was conducted at the vivarium of the Department of General Psychology and the Behavior Analysis Center of Biological Sciences of the State University of Londrina. The rats were housed in polypropylene cages (40 cm x 34 cm x 17 cm) with up to six animals per cage. Water and feed were provided ad libitum throughout the experiment, the vivarium temperature was maintained at approximately 25°C and a 12 hour light/dark cycle was established (light from 7:00 am). The animals' body weight was measured daily before the forced swimming session.

In order to study the effects of chronic forced swimming, chronic fluoxetine treatment and an immunization protocol, roughly half of the animals were submitted to chronic forced swimming sessions and the rest were kept in the vivarium. Each of these groups was subdivided and treated chronically with fluoxetine or saline. Again, each of the four groups was subdivided with part of the animals submitted to the immunization protocol and the other part not. Thus, the following eight groups were involved in the procedure: control saline not immunized (Ctl-Sal-n-Im, n=10); control saline immunized (Ctl-Sal-Im, n=10); control fluoxetine not immunized (Ctl-Fxt-n-Im, n=9); control fluoxetine immunized (Ctl-Fxt-Im, n=9); swimming saline not immunized (Swm-Sal-n-Im, n=10); swimming saline immunized (Swm-Sal-Im, n=10); swimming fluoxetine not immunized (Swm-Fxt-n-Im, n=7); swimming fluoxetine immunized (Swm-Fxt-Im, n=7).

The experimental procedures were approved by the Ethics Committee on Animal Experimentation of the State University of Londrina, Project No. 6977, Case No. 16828/2010.

2.2 Protocol of forced swimming

The forced swimming model was performed in accordance with Lucki (1997) to evaluate the acute effect. In the current study, forced swimming sessions were performed daily for twenty-five days and the behavior of the animals was rated on the first and last day. Forced swimming was performed in a black plastic cylinder (50 cm high and 22 cm in diameter) in which the water was 30 cm deep and kept at 25 ± 2°C. The sessionss were performed individually for 15 minutes between 12 and 2 pm. At the end of the session, each animal was removed from the cylinder and dried. The cylinder was cleaned and the water replaced between use by different groups.

2.3 Fluoxetine: Dilution and application

We used the drug Daforin® (fluoxetine hydrochloride 20mg/ml) diluted 1:2 in saline solution for the experiment. Thirty minutes after the end of each forced swimming session, the animals received 10 mg/kg/day of fluoxetine or saline intraperitoneally (i.p.). The injections began at the first session (pretest) and finished on the penultimate day of the experiment (the 24th day).

2.4 Behavioral evaluation

For behavioral analysis, the animals were filmed during the first five minutes of the 1st and the 25th session of forced swimming. After the tests, the videos were stored on a computer for further analysis.

The amount of time the animals spent in the following behaviors was recorded: floating (complete immobility or faint movements, i.e., the minimum necessary to keep the nose/head above the surface), climbing (vigorous movements with forepaws above the surface or against the cylinder wall) and swimming (horizontal movement without the front legs breaking the surface of the water). The behavioral data were recorded by a trained observer (minimal intra-observer agreement: 0.85).

2.5 Blood collection and immunization

On days 5, 10 and 25 of the study at the end of the forced swimming session, all animals were sedated by non-lethal inhalation of ethyl ether and approximately 1 mL of blood was collected by cardiac puncture. The collected blood was stored in 1.5 ml plastic tubes containing 50 µL of 5% EDTA. On days 5 and 20 the animals belonging to subgroups Ctl-Sal-Im, Ctl-Fxt-Im, Swm-Sal-Im and Swm-Fxt-Im, were inoculated i.p. with a 250 µl solution of 2.5% SRBC.

2.6 Preparation of antigen

The following protocol was used to extract proteins from sheep erythrocytes: the sheep red blood cells were centrifuged in test tubes at a speed of 1000g for 15 minutes. The cell pellet

was then suspended in saline, centrifuged at 1000g for 15 minutes and the leukocyte layer was removed (this process was repeated twice more). After the third wash, the supernatant was removed and 30 ml of Tris-EDTA [5 mM buffer 2-Amino-2-hydroxymethyl-propane-1,3-diol/hydrochloric acid (Tris-HCl), pH 7.6, containing 1 mM Ethylenediamine tetraacetic acid (EDTA)] was added. The tubes were subjected to centrifugation at 25000g for 30 minutes (this process was repeated until the supernatant had turned pink). The contents of the tubes were then filtered through cheesecloth and underwent a final wash with Tris-EDTA. The pellet obtained was suspended in 0.1% Sodium Dodecyl Sulfate (SDS) in Phosphate Buffered Saline (PBS) at a volume three times that of the pellet. The suspension was dialyzed for 24 hours at room temperature and the PBS/SDS solution was changed at least twice. Aliquots of the suspension were stored at -20°C. The protein suspension dosage followed Bradford (1976).

2.7 Sacrifice

On the 25th day of study, after finishing the forced swimming test, the animals were again non-lethally sedated by inhalation of ethyl ether for blood collection, after which the animals were sacrificed by lethal ethyl ether inhalation. The spleen, thymus and adrenal glands of each rat were subsequently removed to assess the relative weight.

2.8 ELISA

To assess the production of antibodies (IgM, IgG1 and IgG2a), an enzyme-linked immunosorbent assay (ELISA) containing 100 µl of a solution of 2.5 mg/ml sheep erythrocyte proteins obtained in the above-described manner was added to each well. The plasma was diluted 1:100. The dilutions of peroxidase conjugated anti-IgM, anti-IgG1 (Zymed) and anti-IgG2a (BETHYL) were 1:10000, 1:20000 and 1:5000, respectively.

ELISA was conducted according to the following protocol: first, the 96-well plates were coated with 100 µl of the antigen diluted in carbonate-bicarbonate pH 9.6 and incubated overnight at 4°C. The plates were then washed 3 times with PBS-Tween 0.05% and blocked with 150 µl of PBS with skim milk (PBS-milk) 5% in each well for 1 h at 25°C. After 3 washes with PBS-Tween 0.05%, plasma samples diluted in PBS-milk 1% (100 µl of 1:100 diluted sample per well) were incubated for 1 h at 25°C. The plates were then washed 3 times with PBS-Tween 0.05% and the conjugate (100 µl of conjugate diluted in PBS-milk 1% per well) anti-IgM, anti-IgG1, or anti-IgG2a was incubated for 1 h at 25ºC, washed 3 times with PBS-Tween 0.05%, and then the substrate (sodium acetate buffer 0.1 M pH 5, containing TMBZ – tetramethylbenzidine of 1% and H_2O_2 - hydrogen peroxide 0.005%) was added (100 µl of substrate/well). After incubation in the dark for 15 minutes at 25°C, 50 µl of 1N H_2SO_4 was added per well. Reading was performed in a microplate reader at 450 nm.

2.9 Statistical analysis

Statistical analysis was performed with Statistica 5.0®. To evaluate homogeneity and normality, the Levene and Kolmogorov-Smirnov tests were used. To evaluate antibody production (IgM, IgG1 IgG2a), four-way repeated-measures ANOVA was performed including the effects of the swimming sessions (Ctl X Swm), fluoxetine treatment (sal X fxt), immunization (n-Im X Im) and repeated measurement factor of blood sampling time

(preImmunization X after the 1st immunization X after the 2nd immunization). Behavioral comparisons were also performed by means of four-way ANOVAs, but with a different repeated-measures factor (Session 1 x Session 25). Repeated-measures comparisons of the following masses were conducted: body (fluctuation), spleen, adrenal gland and thymus. Therefore, the above described remaining factors were analyzed in three-way ANOVAs run for this purpose. When interactions of main effects were found to be significant, Tukey post hoc tests were applied. The significance level was set at $P < 0.05$.

3. Results

Figure 1a shows the production values of IgM antibody groups. The results show no effects for stress (F [1.64] = 0.348, P> 0.05), but effects for immunization (F [1.64] = 20.050, P <0.001), drug (F [1.64] = 6.673, P <0.05), time (F [2.128] = 32.208, P <0.001), interaction between immunization and time (F [2.128] = 21.710, P <0.001), drug and time (F [2.128] = 7.383, P <0.001) and immunization, drug and time (F [2.128] = 9.268, P <0.001). Comparing the pre, post1 and post2 immunization periods, the Tukey test showed that there was an increase in IgM production only for the Ctl-Sal-Im and Swm-Sal-Im groups. We observed that only animals treated with saline responded to inoculation with SRBC, while fluoxetine inhibited the production of antibodies.

The production of IgG2a antibody (Figure 1b) appeared to be similar to the values observed for IgM. Four-way ANOVA showed no stress effect (F [1.64] = 1.188, P> 0.05), but effects for immunization (F [1.64] = 26.326, P <0.001), drug (F [1.64] = 7.139, P <0.05), time (F [2.128] = 25.483, P <0.001) , immunization and drug interaction (F [1.64] = 7.814, P <0.01), immunization and time (F [2.128] = 25.734, P <0.001), drug and time (F [2.128] = 6.578, P < 0001) and immunization, drug and time (F [2.128] = 6.630, P <0.01). In the pre, post1 and post2 immunization periods, the Tukey test showed increased production of IgG2a only in Ctl-Sal-Im and Swm-Sal-Im. Only non-stressed animals treated with saline responded to inoculation with sheep red blood cells, while fluoxetine inhibited the production of antibodies.

Figure 1c shows the production values for IgG1 antibody. There were no effects for stress (F [1.64] = 0.404, P> 0.05) drug (F [1.64] = 0.001, P> 0.05), but effects for immunization (F [1.64] = 48.908, P <0.001), time (F [2.128] = 81.116, P <0.001), interaction between stress and drug (F [1.64] = 9.370, P <0.01), immunization and time (F [2.128] = 67.428, P <0.001), stress, immunization and drug (F [1.64] = 11.223, P <0.01), stress, drug and time (F [2.128] = 18.953, P <0.001) and stress, immunization, drug and time (F [2.128] = 20.187, P <0.001). Comparing the pre, post1 and post2 immunization periods, an increase in IgG1 production was observed only for the Ctl-Sal-Im and Swm-Fxt-Im groups. It was observed that stress and fluoxetine in isolation inhibit the production of IgG1, but that stress and drugs together interacted to cause antibody production similar to that of the control group (Ctl-Sal-Im).

The variation in rat body mass was not altered by immunization (F [1.64] = 0.34, P> 0.05), although stress (F [1.64] = 19.948, P <0.001) and drug effects (F [1.64] = 111.595, P <0.001) were observed. There was no significant interaction between variables. Intergroup comparison revealed that fluoxetine was responsible for reducing body mass (Figure 2).

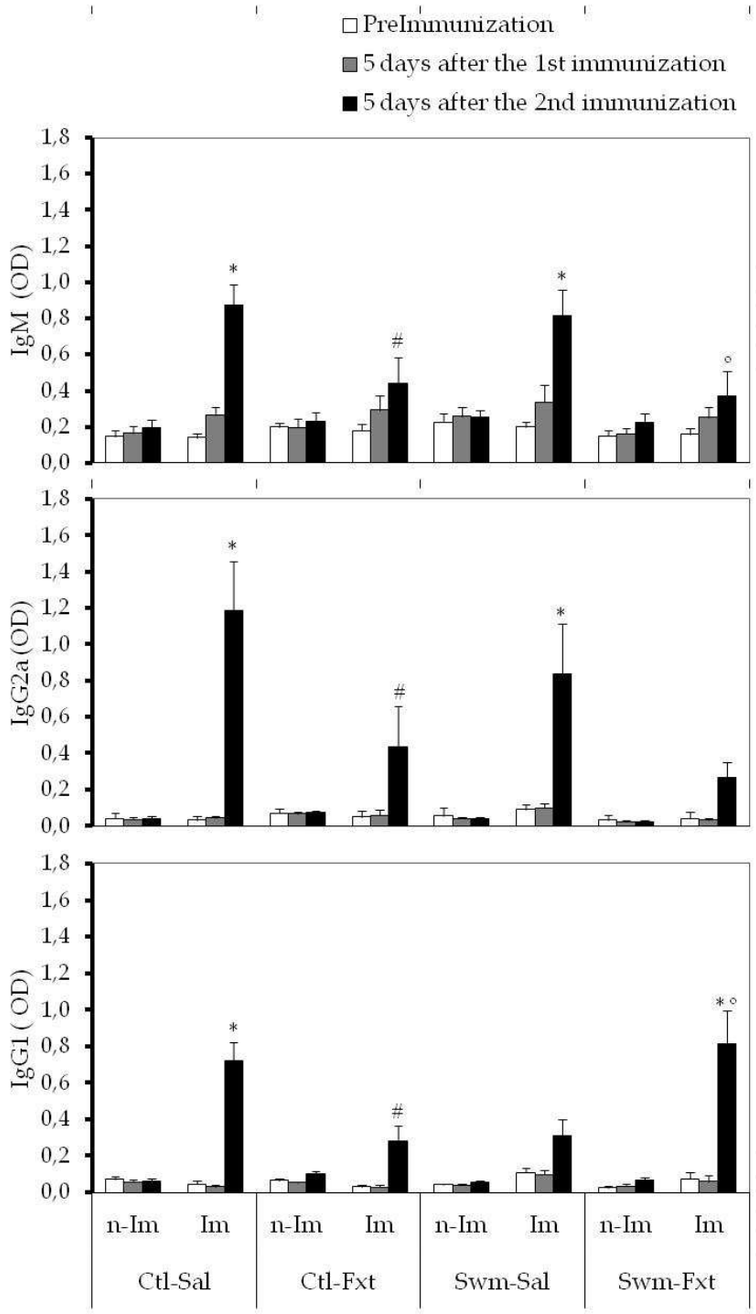

Fig. 1. Variation (mean ± SEM) in the production of antibody. We analyzed the variation in the production of antibodies (IgM, IgG2a and IgG1) at three different points in time (pre-immunization, five days after the first immunization and 5 days after the second immunization). Fluoxetine was responsible for suppressing the production of IgM (a) and IgG2a (b). In relation to IgG1 (c), the administration of only stress and fluoxetine impaired antibody production. However, the interaction between these variables did not impair production. * Different the pre-immunization and 5 days after the first immunization ($P < 0.001$); #Different from Ctl-Sal-Im 5 days after the second immunization ($P < 0.001$); º Different Swm-Sal-Im 5 days after the second immunization ($P < 0.002$).

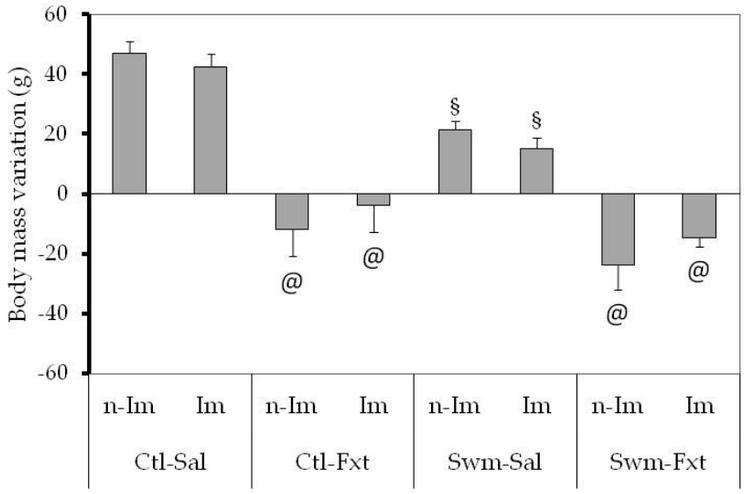

Fig. 2. Variation (mean ± SEM) in body mass. It was observed that both fluoxetine and swimming resulted in reduced body mass. @ Different the saline group that underwent the same treatment (P < 0.05); § Different from the control group that underwent the same treatment (P < 0.05).

There was no stress (F [1.64] = 2.660, P> 0.05) or immunization effect (F [1.64] = 0.373, P> 0.05) on the relative mass of the adrenal glands. There was a significant effect for drug (F [1.64] = 38.558, P <0.001) and interaction between drugs and immunization (F [1.64] = 2.479, P <0.05). The Tukey test showed an increase in relative mass of the adrenal group Swm Fxt-n-Im compared to its control Swm-Salt-n-Im (Table 1).

There was no stress effect on the relative mass of the spleen (F [1.64] = 0.728, P> 0.05), but there was a drug effect (F [1.64] = 19.534, P <0.001, Table 1). Nevertheless, there was no significant difference between groups in post hoc comparisons.

There was no stress (F [1.64] = 0.276, P> 0.05) or immunization effect (F [1.64] = 0.704, P> 0.05) on relative thymus mass, but a drug effect (F [1.64] = 32.504, P <0.001) and an interaction between stress and drug (F [1.64] = 7.535, P <0.05) was detected. It was observed that the drug reduced the relative mass of the thymus in unstressed animals treated with fluoxetine (Table 1).

Organ	Control				Swim			
	Saline		Fluoxetine		Saline		Fluoxetine	
	n-Im	Im	n-Im	Im	n-Im	Im	n-Im	Im
Adrenals	6.6 ± 0.6	7.6 ± 0.3	9.7 ± 0.7	10.0 ± 0.8	6.9 ± 0.8	7.7 ± 0.8	13.1 ± 1.6	9.8 ± 0.5
Spleen	158.4 ± 3.4	150.4 ± 5.6	222.2 ± 27.7	206.8 ± 26.8	143.4 ± 5.2	164.0 ± 7.6	202.0 ± 20.5	189.5 ± 18.0
Thymus	66.2 ± 4.3	71.5 ± 3.8	36.0 ± 7.3	36.1 ± 5.0	54.9 ± 5.1	57.4 ± 5.7	42.1 ± 7.9	47.2 ± 3.4

Table 1. Relative mass of the adrenal glands, spleen and thymus of rats at the end of the experiment. It was observed that fluoxetine was responsible for changing the relative mass of the three organs analyzed, with the adrenal glands and thymus increased and the spleen reduced (P <0.05). Measure (1 = 0.001% of the body mass).

Figure 3a shows the duration of floating behavior. Statistical analysis showed no effects for immunization (F [1.30] = 0.078, P> 0.05) or drug (F [1.30] = 1.099, P> 0.05) but effects for time

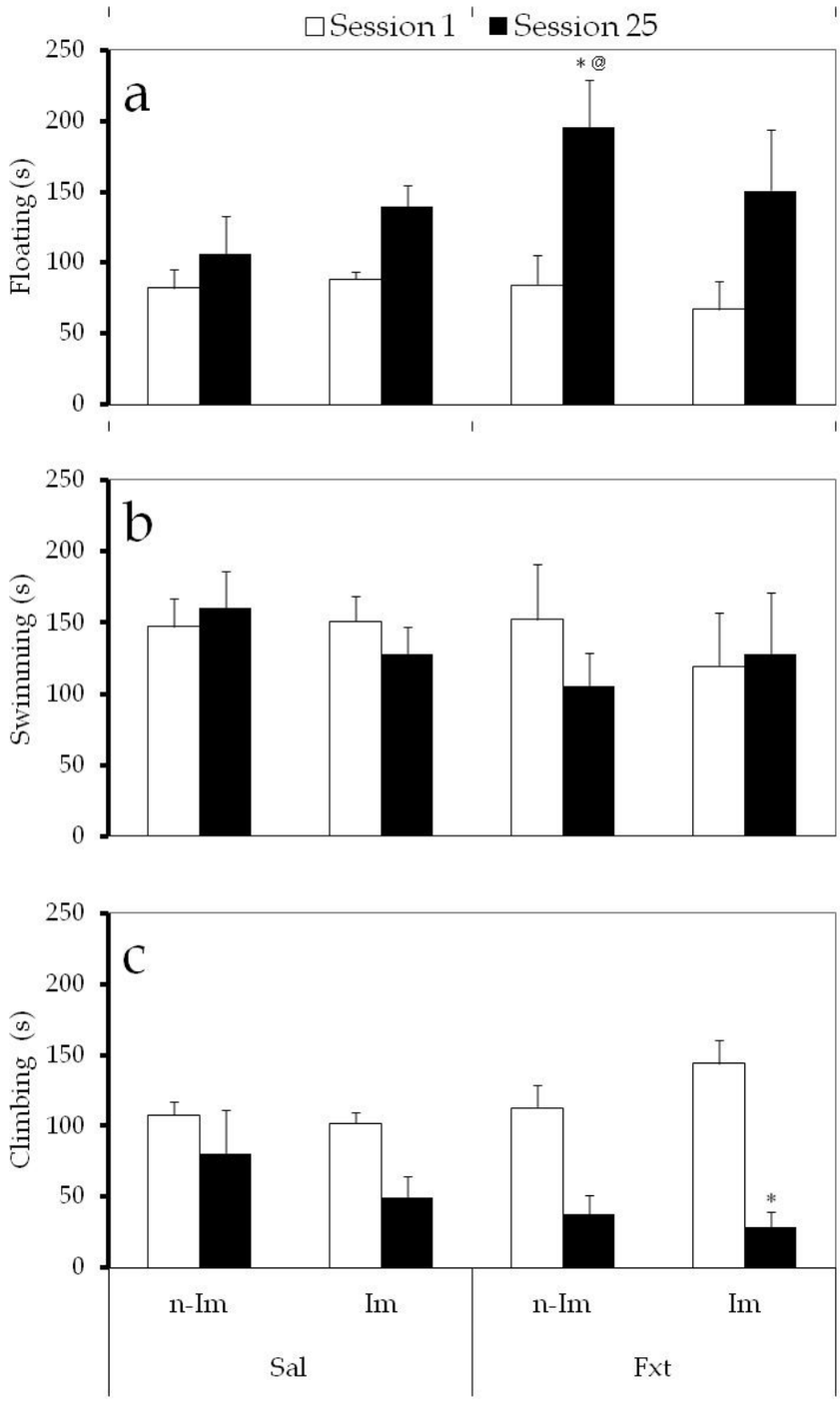

Fig. 3. Variation in the time of analyzed behaviors. Fluoxetine treatment increased floating (a) and reduced climbing (c) behavior between Session 1 and 25; no alteration was found in swimming behavior (b). The animals treated with saline did not show significant alterations in behavior between sessions. *, significant difference compared to Session 1 ($P < 0.01$). @, significant difference in the same session compared to the saline group that had been otherwise submitted to the same treatment ($P < 0.05$).

(F [1. 30] = 30.010, P <0.001). An interaction between factors occurred only with drug and time (F [1.30] = 5.989, P <0.05). Comparing the 1st and the 25th session, a reduction was observed only in the nonimmunized, drug treated group. There was also a distinction observed between the Fxt-n-Im and Sal-n-Im groups at the 25th session.

For swimming, statistical analysis revealed no effects for immunization (F [1.30] = 0.208, P> 0.05), drug (F [1.30] = 0.861, P> 0.05), time (F [1.30] = 0.563, P> 0.05) or interaction of factors (Figure 3b).

Figure 3c shows the time of analyzed behaviors. There were no effects for immunization (F [1.30] = 0.081, P> 0.05) or drug (F [1.30] = 0.091, P> 0.05) and effects for time (F [1. 30] = 32.243, P <0.001). There was an interaction between drug and time (F [1.30] = 5.338, P <0.05). Comparing the 1st and 25th sessions, an increase in climbing time was detected in the Fxt-Im group.

4. Discussion

The current study investigated the effects of chronic stress and the administration of the drug fluoxetine on humoral immune response. It assessed primary and secondary immune response against sheep red blood cells, variation in body mass and the relative mass of the adrenal glands, thymus and spleen, as well as the behavior of rats subjected to a daily forced swimming protocol, which is an model used to assess depression-like behavior in rodents.

In general, stress is considered to be an immunosuppressant. Elenkov & Chrousos (1999) conducted an extensive review on the influence of stress on the immune system and found that acute stress produced subacute or chronic immunosuppressive activity on cellular immune response. On the other hand, stress also was found to have an immunostimulating effect on humoral immune response. Another literature review Segerstrom & Miller (2004) that included research from the last 30 years on the effects of stress on immune function in men and women found no relationship between acute and subacute stress regarding modulation of humoral immune response. Nevertheless, it was observed that stress is associated with chronic immunosuppression in that it lowered antibody capacity against an influenza virus.

The ability of stress to inhibit cellular immune response (Th1) is probably related glucocorticoid and catecholamine suppression of pro-inflammatory cytokines, IL-12, IFN-γ and TNF-α (Elenkov & Chrousos, 1999). Regarding the suppression of cellular immune response, several studies have shown that stress can cause a predisposition to autoimmune diseases (rheumatoid arthritis and type 1 diabetes), allergies (asthma, food allergies and emphysema), and some types of cancer, including Kaposi's sarcoma and Epstein-Barr virus associated B-cell lymphomas (Reiche et al., 2004).

On the other hand, the modulation of humoral immune response by stress is a controversial topic in the literature because studies differ regarding the possible modulation. Baldwin et al. (1995) submitted rats to a stress regime that can be considered subchronic (forced swimming for 3-5 days, 60 minutes each session) and found no differences in the production of anti-sheep red blood cells between stressed and unstressed rats. Besides studies that have found no increase, others have observed a decrease. Kennedy et al. (2005) submitted rats to acute restraint stress and found that it did not alter the production of IgG1 (Th2) but suppressed the production of IgM and IgG2a (Th1) antibodies. Stanojevic et al. (2003)

verified the effects of shock stress for five days in rats and, after immunization with bovine serum albumin (BSA), found that there was suppressed production of IgG anti-BSA compared to controls upon second exposure to the antigen. Hawley et al. (2006) showed that stress caused by high social competition in birds involves a lower production of anti-sheep erythrocytes. Rammal et al. (2010) found that anxious mice produced fewer IgA and IgE antibodies than their nonanxious counterparts, and when both groups were subjected to restraint stress, it appears that all studied antibodies (IgA, IgE and IgG) were suppressed in both groups. On the other hand, Guéguinou et al. (2011) analyzed the natural antibodies of mice subjected to a rotational velocity model (2 and 3 G-force) for 21 days and found an increase in IgG levels of animals subjected to 2Gs. Thus, it can be inferred that the type and length of exposure to the stressor has a direct relationship with the modulated production or elimination of certain antibodies.

In our study, the chronic stress of forced swimming did not interfere in the production of antibody classes IgM and IgG2a, although the production of IgG1 was suppressed. These results are similar to those of Kennedy et al. (2005). The modulation of IgG1 antibody production in mice suggests a suppression of the Th2-type response, which in rats is associated with the production of antibodies to this class of immunoglobulins. On the other hand, the results suggest that the Th1 immune response is not affected by forced swimming since we did not observe a change in the levels of IgG2a antibodies. It is important to note that the production of antibodies in response to an antigen derived from a complex network of cellular interactions that involve the production of molecules with opposite effects, such as cytokine IFN-γ in mice, which has a stimulating effect on cellular immune response and IgG2a antibody production as well as an inhibiting effect on humoral immune response and the production of IgG1 antibody, whereas IL-4 has the opposite effect. The fact that the forced swimming model results in the removal of IgG1 antibodies from production suggests that, by mechanisms not yet understood, stress results in the modulation of signals involved in Th2 response without changing the Th1 response. Whereas there is an antagonistic relationship between IFN-γ and IL-4, these results suggest that the stress-modulated molecular mechanism does not directly involve the main molecules responsible for modulation of antibody production. Recent studies have shown that the role of neurotransmitters in immune system function may be more important than previously considered (Rosas-Ballina et al., 2011).

Besides the relationship between stress and humoral immune response, we investigated the action of fluoxetine on this relationship. Although the 25 days of forced swimming in the present study did not affect the normal production of IgM or IgG2a but inhibited IgG1, we can speculate that the chronic use of this model may stimulate cellular immune response. The administration of fluoxetine inhibited the production of all immunoglobulin classes studied, which shows its general immunosuppressive effect, both for Th1 and Th2. However, the interaction of forced swimming x fluoxetine normalized the production of IgG1. This suggests that stress alone diverts the immune response to Th1-type, while fluoxetine alone has an immunosuppressive effect on humoral immune response. On the other hand, administration of fluoxetine in animals subjected to forced swimming can modulate the immune response to a Th2 pattern. A study about the effects of fluoxetine on humoral immune response showed that mice with rheumatoid arthritis that were treated with fluoxetine (10 or 25 mg/kg/day) for seven days had no changes in the levels of anti-collagen antibodies (IgG1 and IgG2a) (Sacre et al., 2010). This result is at odds with the

findings of this study since the time/effect analysis of fluoxetine showed immunosuppression of all studied classes of antibodies after twenty-four days of treatment. These results suggest that the effect of fluoxetine depends on the physiological state of the animal. It is important to note that fluoxetine administered concomitantly with stress can have an immunostimulatory effect. Frick et al. (2009) observed that chronic restraint stress in rats causes decreases in CD4 + T lymphocytes and no change in CD8 + T lymphocyte but when treated with fluoxetine, initial values of CD4 + T cells were restored. According to Freire-Garabal et al. (1997), stressed rats treated with fluoxetine had a higher number of circulating lymphocytes than their control counterparts (stressed and not treated with fluoxetine).

The reduction in specific antibody levels observed in our study is probably related to the action of fluoxetine on the production of cytokines and B lymphocytes, the cells responsible for producing antibodies, as has been observed in other studies. Kubera et al. (2000) demonstrated that the administration of fluoxetine for more than four weeks suppresses the production of IL-4, the main stimulus for differentiating T helper cells into Th2 cells. The decrease in Th2 production may influence isotype synthesis or immunoglobulin levels. Regarding the plasma level of antibodies, Laudenslager & Clarke (2000) observed an increase in immunoglobulin class IgM and IgG and a decrease in the levels of specific IgG antibodies against the tetanus toxoid immunogen in monkeys (*Macaca mulatta*). Therefore, fluoxetine can induce an increased level of total Ig and a decreased level of specific antibodies. However, Sluzewska et al. (1995), studying depressed patients treated with fluoxetine, showed a decrease in IL-6, the cytokine responsible for the growth of B lymphocytes, which differentiate into antibody producers. Moreover, Genaro et al. (2000) observed that fluoxetine had an inhibitory effect on the proliferation of B lymphocytes that had been stimulated by LPS.

The immunosuppressive action of fluoxetine cannot be restricted to the production of antibodies. Pellegrino & Bayer (2002) observed that the in vitro proliferation of lymphocytes from rats that had received fluoxetine via i.p. (5 mg/kg) was lower than their respective controls, suggesting that the antidepressant has an immunosuppressive role for lymphocytes. Fazzini et al. (2009) found that three weeks of continued fluoxetine use in rats triggered an increase in CD8 + T lymphocytes and reduced CD4 + T cells.

The immunomodulatory action of fluoxetine probably involves the participation of cytokines. Patients with major depression have high levels of IL-6, and treatment with fluoxetine for 8 weeks leads to normalization of the cytokine levels (Nishida et al., 2002). Frick et al. (2008), studying cancerous rats, observed that fluoxetine treatment has a direct relationship with increased production of anti-tumor cytokines (IFN-γ and TNF-α), which resulted in lower rates of tumor growth and, therefore, longer survival time. On the other hand, Roumestan et al. (2007) found that fluoxetine had an anti-inflammatory effect (5, 10, 15 and 20 mg/kg) when rats were treated thirty minutes prior to inoculation with LPS and reported reductions of 60% in TNF-α levels and 50% in mortality compared to controls. Sacre et al. (2010) also observed that fluoxetine had an anti-inflammatory effect in rats with rheumatoid arthritis that were treated with 25 mg/kg for seven days, as reflected in reduced levels of IL-12 and joint damage. On the other hand, some studies have failed to show a relationship between fluoxetine and the modulation of cytokine production (Kubera et al., 2004; Maes et al. 1995; Jazayeri et al., 2010). Grundmann et al. (2010) treated rats orally with 10 mg/kg/day of fluoxetine for 21 days and observed no changes in the production of proinflammatory cytokines (IL-6 and TNF-α).

The production of pro- and anti-inflammatory cytokines due to stress plus fluoxetine is dependent on the type of stress and route of drug administration. Sprague-Dawley strain rats, after 21 days of restraint stress and chronic oral treatment with fluoxetine (10 mg/kg), showed lower production of IL-6 than stressed-only animals, although TNF-α levels increased, reaching values similar to those of untreated stressed animals (Grundmann et al., 2010). On the other hand, Kubera et al. (2006) pre-treated rats with imipramine (5 mg/kg) 1, 5 and 24 hours before forced swimming and found that the splenocytes of treated animals produced more IL-10 than controls (stressed and treated with vehicle), with no IFN-γ differences observed in any group. Rogoz et al. (2009) treated rats 1, 5 and 24 hours before forced swimming with 10 mg/kg of fluoxetine i.p. and observed that the interaction between stress and fluoxetine did not alter the splenocyte production of IL-10 or IFN-γ.

There are reports that the chronic administration of fluoxetine either causes weight loss (Wellman et al., 2003) or prevents weight gain (Gutierrez et al., 2002). In the present study, the chronic administration of fluoxetine led to a reduction in body mass when compared with saline treatment; this reduction was more pronounced when the animals were treated with the drug and subjected to forced swimming. First et al. (2011) treated rats for five weeks with fluoxetine (5 mg/kg) and observed reduced body mass. However, when they were treated with the drug and submitted to chronic stress with multiple stressors, fluoxetine prevented weight loss due to this protocol. Zafir & Banu, (2007) also observed weight maintenance by chronic administration of fluoxetine in animals subjected to restraint stress. It is important to point out that the above-mentioned studies differed in the degree of stress generated. In this study 15 min/day of forced swimming did not prevent the animals from gaining weight, but First et al. (2011), using various types of stressors for five weeks and Banu & Zafir (2007), using four hours of restraint stress, observed reduced body mass. Thus, combined multiple stressors or prolonged restraint seem to be more stressful than forced swimming. Considered jointly, these studies indicate two seemingly opposite effects of fluoxetine: in the presence of severe stressors known to induce mass reduction, the drug prevents such losses, while in the presence of mild stressors, the drug leads to weight loss, which suggests an anorexic effect. Human studies have confirmed the anorectic effect of fluoxetine in that reductions in body mass from the chronic administration of fluoxetine were observed in obese individuals (Wise, 1992).

Stress affects the mass of the adrenal glands and lymphoid organs such as the thymus and spleen. Baldwin et al. (1995) investigated the effects of forced swimming (3 to 5 days, sixty minutes per session) and found that the number of rats housed together (one or five) influenced the relative masses of the adrenal glands, spleen and thymus, the production of corticosterone and body mass. They observed that forced swimming, regardless of the type of accommodation, reduced the spleen, thymus and body mass of animals, but did not alter the production of corticosterone or the relative mass of the adrenal glands. When the animals were subjected to social isolation and forced swimming, however, there was increased corticosterone production and adrenal mass in addition to the above-mentioned effects, showing that these two models administered separately do not lead to stress, but together are stressful. Regarding the chronic effect of forced swimming, Zivkovic et al. (2005a) found that after submitting rats to 21 days of this protocol, the thymus weight of stressed animals was lower than that of non-stressed animals. In another study by the same authors (2005b), blood was collected from rats after their final swimming session for analysis of circulating corticosterone levels and it was observed that, even after 21 days of chronic forced swimming, corticosterone values remained high.

In our study, the adrenal gland mass of Wistar rats submitted to swimming (15 min daily for 25 days) did not change, which was a further similarity with the findings of Baldwin et al. (1995), i.e., body mass reduction in animals submitted to swimming. Our study differed from the above-mentioned studies in that our stress model did not lead to changes in spleen or thymus mass. Moreover, Connor et al. (1998) observed no changes in Sprague-Dawley spleen weight after acute forced swimming, which shows that, depending on the strain and stress time, body mass values may or may not vary.

Fluoxetine is also responsible for changing the mass of the adrenal glands, spleen and thymus of rodents. Garabal-Freire et al. (1997) submitted mice to a sound stressor (100 dB, 1 to 3 hours per day, four to twelve days) and observed a decrease in the number of thymic and spleen cells; this stressor also contributed to a reduction in relative thymus weight, a condition reversed by treatment with fluoxetine (5 mg/kg). Kubera et al. (2006) treated rats with three doses of imipramine 1, 5 and 24 hours before forced swimming and found that acute treatment with this drug did not alter the relative thymus weight, but did reduce spleen weight. In the present study, 24 days of fluoxetine treatment (10 mg/kg) reduced the relative thymus weight and body mass of rats and increased spleen and adrenal gland mass. Thus, either chronic treatment with fluoxetine stressed the animals or the change in relative adrenal mass is merely a reflection of the change in body mass since the adrenal glands did not necessarily increase. However, the sudden loss of body mass would have led to this apparent increase.

The currently-used antidepressants have specific compounds that act on different regions of the central nervous system, so it is expected that their use in rats or mice would lead to improvement in depression symptoms, i.e., reduced time floating (passive behavior) and increased time climbing and/or swimming (active behavior) during a forced swimming stressor (Piras et al., 2010). However, increases in climbing and/or swimming are dependent on the type of drug administered (Cryan & Lucki, 2000). Page et al. (1999) observed a reduction in floating time and an increase in swimming time in rats treated with fluoxetine. Carr et al. (2010) used fluoxetine in rats (20 mg/kg) three times before forced swimming yielded similar results. Cryan & Lucki (2000) compared fluoxetine and reboxetine in a rat forced swimming model and found that both drugs led to reduced flotation time, although the former increased swimming time and the latter increased climbing time.

To investigate the effects of chronic treatment with fluoxetine (10 mg/kg), Hansen et al. (2011) treated Wistar rats for 48 days with subcutaneous injections, after which the animals were subjected to forced swimming. The results showed that the floating, swimming and climbing times of treated rats were similar to those observed when fluoxetine was administered 24 hours before the test (acute effect). Pedreañez et al. (2011), studying the effects of forced swimming as a chronic stressor, carried out fifteen 30-min forced swimming sessions and found that the animals' active behavior dropped by 84 percent between the first and last session.

In our experiment, forced swimming was performed over a chronic period (twenty-five days). The results were expected to be similar to those in the literature (with test-retest separated by 24 h) or to those of Pedreañez et al. (2011) for rats subjected to chronic swimming. We found that, after the chronic treatment period, the time values were different from those observed when the test and retest were separated by 24 h. Stressed animals treated with saline had no alterations in floating, swimming or climbing times, indicating

that forced swimming, when performed on a chronic basis, is not an appropriate model for investigating behavioral changes in rats. It should be pointed out that different strains of rats exhibited different behavior in the two studies: the active behavior of Sprague-Dawley rats was reduced in Pedreañez et al. (2011) whereas, in the present study, Wistar behavior was constant throughout the protocol.

On the other hand, it was observed that animals treated with fluoxetine significantly increased floating time after treatment. Since climbing time was also reduced, we can infer that adaptation to the drug also leads to behavioral changes, contradicting expected behavior for this model of stress/depression. Two possibilities could explain this: first, chronic treatment with fluoxetine engenders passive behavior; second, their reduced body weight made them denser, which may have facilitated buoyancy and thus reduced effort expenditure.

5. Conclusion

We observed in this study that animals treated with fluoxetine and submitted to a 25 day forced swimming protocol had a reduced production of IgM and IgG2a and an increased production of IgG1. Considering the unique effect of the drug, the adrenal gland and relative spleen weight increased, while thymus weight was reduced. Drug-treated rats lost body mass compared to saline-treated rats. Regarding the analyzed behaviors, treatment with fluoxetine resulted in distinct changes in acute effect, indicating that swimming is not be trusted as a model chronic stressor in rats. However, the alterations observed in this study may have important implications for the treatment of depression in humans since fluoxetine appears to impair the production of antibodies. Thus the indiscriminate use of this drug for non-stressed individuals must be questioned.

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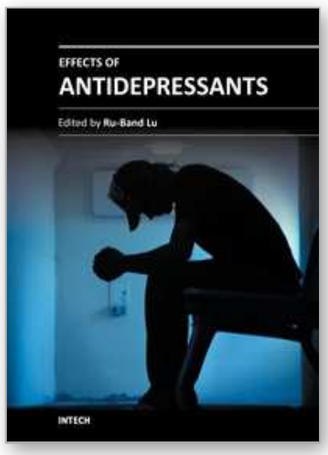

Effects of Antidepressants

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Over the last fifty years, many studies of psychiatric medication have been carried out on the basis of psychopharmacology. At the beginning, researchers and clinicians found the unexpected effectiveness of some medications with therapeutic effects in anti-mood without knowing the reason. Next, researchers and clinicians started to explore the mechanism of neurotransmitters and started to gain an understanding of how mental illness can be. Antidepressants are one of the most investigated medications. Having greater knowledge of psychopharmacology could help us to gain more understanding of treatments. In total ten chapters on various aspects of antidepressants were integrated into this book to help beginners interested in this field to understand depression.

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