

# Involvement of Estrogen Receptors in the Anxiolytic-Like Effect of Phytoestrogen Genistein in Rats with 12-Week Postovariectomy

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## ABSTRACT

Phytoestrogens are natural compounds found in some vegetables, and they replicate many of the physiochemical and physiological properties of estrogens, including the regulation of mood. The phytoestrogen genistein exerts anxiolytic-like effects in rats with a chronic absence of ovarian hormones, but the mechanism involved in this effect remains to be explored. The present study explored the participation of estrogen receptor- $\beta$  in the anxiolytic-like effect of genistein (1.0 mg/kg, i.p., for 4 days) in Wistar rats with 12-week postovariectomy, considered as experimental model of postsurgical menopause. In the light/dark test, a useful tool for anxiety study and for the screening of anxiolytic drugs, genistein reduced the latency to enter and increased the time spent in the light compartment and significantly increased the frequency and time spent exploring the light compartment compared with the control group, which is considered as an anxiolytic-like effect at experimental level. All behavioral effects produced by genistein in the light/dark test were blocked by previous tamoxifen administration (5.0 mg/kg, s.c., for 6 days), a non selective antagonist for estrogen receptor- $\beta$ . The effects produced by genistein or tamoxifen in this test were not related to significant changes in general motor activity evaluated in the open field test. In conclusion, the specific contribution of present investigation was identify that estrogen receptor- $\beta$  is involved in the anxiolytic-like effect produced by phytoestrogen genistein in rats with a long-term absence of ovarian hormones; supporting the hypothesis that estrogen receptor- $\beta$  participates in the regulation of anxiety associated with low concentration of ovarian hormones and in the anxiolytic-like effects produced by natural estrogenic compounds such as phytoestrogens.

**Keywords:** Anxiety; Genistein; Phytoestrogen; Estrogen Receptor- $\beta$ ; Light/Dark Test; Tamoxifen

## 1. Introduction

Phytoestrogens are natural chemical compounds abundantly found in fruits, vegetables, legumes, whole grains, and especially flaxseed, clover, and soy products, and they replicate some of the physiochemical and physiological properties of estrogens [1-3]. The phytoestrogens genistein and daidzein, among others, are thought to exert the most potent estrogenic activity at the preclinical and clinical levels [1,4]. These previous studies suggest that phytoestrogens may be utilized in the treatment of physiological and emotional alterations related to low concentrations of ovarian hormones (*i.e.* 17 $\beta$ -estradiol and progesterone), which occur in natural or surgical menopause [5,6]. Some clinical trials suggested that a phytoestrogen-rich diet protects women against age-re-

lated diseases, certain types of cancers, and postmenopausal symptoms, such as osteoporosis, hot flashes, and mood swings [7-10], demonstrating the potential therapeutic effect of phytoestrogen in the management of physical and emotional alterations related to dysregulation of ovarian hormone function.

In preclinical trials, controversy exists with regard to the effects of phytoestrogens on mood. Some reports showed a significant reduction in anxiety-like behavior in rats fed a phytoestrogen-rich diet [11,12], whereas rats fed a low-phytoestrogen diet exhibited increased anxiety-like behavior [13]. Apparently, low phytoestrogen concentrations inhibit aromatase, which blocks the conversion of testosterone to 17 $\beta$ -estradiol, whereas high phytoestrogen concentrations produced estrogen-like actions [14], which would explain the anxiolytic-like effect of phytoestrogen at higher doses.

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Administration of genistein (10 mg/kg/14days) in ovariectomized rats exerted antidepressant-like effects in the forced swim test [15,16]. Additionally, administration of genistein (0.5 and 1.0 mg/kg/4days) in rats with a chronic absence of ovarian hormones exerted anxiolytic-like effects in the light/dark test [17], a common behavioral test used for assessing anxiolytic compounds at the experimental level [18]. Considering evidence that phytoestrogens [19] and particularly genistein [20-22] have a particularly high affinity for estrogen receptor- $\beta$ , is suggested that this substrate-receptor interaction may have a causal role in the anxiolytic-like effect seen. Selective modulators of this receptor (e.g.,  $17\beta$ -estradiol, diarylpropionitrile, and dihydrocoumestrol, among others) exert anxiolytic-like effects at the preclinical level [23,24]. Nonetheless, the participation of estrogen receptor- $\beta$  in the anxiolytic-like effect of genistein in rats with a chronic absence of ovarian hormones produced after 12-week postovariectomy remains to be explored.

In the present study, we hypothesized that the anxiolytic-like effect of genistein in ovariectomized rats is established by an interaction with estrogen receptor- $\beta$ . Therefore, we assessed the effect of pretreatment with tamoxifen, a non selective estrogen receptor- $\beta$  antagonist that readily crosses the blood-brain barrier [25], on the anxiolytic-like effect of genistein in the light/dark test in rats with 12-week postovariectomy.

## 2. Materials and Methods

### 2.1. Animals

Thirty-two ovariectomized adult female Wistar rats, weighing 200 - 250 g at the beginning of the experiments, were used. The rats were housed in Plexiglas cages (eight rats per cage) under a 12/12 h light/dark cycle (light on at 6:00 AM) at an average temperature of 25°C ( $\pm 1^\circ\text{C}$ ) with *ad libitum* access to purified water and food (Teklad lab animal diets, Harlan Co.). All of the experimental procedures were performed according to the Guide for the care and use of laboratory animals published by the National Institutes of Health [26] and the Norma Oficial Mexicana para el Cuidado y Uso de Animales de Laboratorio [27]. All efforts were made to reduce the number of animal and suffering during experiments.

### 2.2. Ovariectomy

Ovariectomy was performed according to previous studies [17,28]. After the surgery and to ensure the long-term absence of ovarian hormones and high levels of anxiety behavior, the rats were returned to the housing facilities for 12 weeks [29]. After this time period, the rats were randomly assigned to each of the experimental groups and subjected to the treatments and behavioral tests.

### 2.3. Experimental Groups and Treatments

The rats at 12 weeks postovariectomy were assigned to four independent groups (eight rats per group). The treatment conditions included four combinations: vehicle-vehicle, tamoxifen-vehicle, vehicle-genistein, and tamoxifen-genistein. The first treatment, tamoxifen (5.0 mg/kg) or its vehicle (corn oil), was administered subcutaneously for 6 consecutive days before the behavioral tests that began 11 weeks postovariectomy. The second treatment, genistein (1.0 mg/kg) or its vehicle (35% 2-hydroxypropyl- $\gamma$ -cyclodextrin solution), was administered intraperitoneally for 4 consecutive days before the behavioral tests, which began simultaneously on day 3 of tamoxifen or vehicle treatment. All treatments were administered every 24 h (volume injected: 1.0 mL/kg). Thirty minutes after the last administration of genistein or its vehicle, the rats were evaluated in the light/dark test and subsequently in the open field test. Genistein (minimum 98% HPLC), tamoxifen (minimum 99%), and 2-hydroxypropyl- $\gamma$ -cyclodextrin were acquired from Sigma-Aldrich Co. (St. Louis, Missouri, USA). Corn oil was acquired from Fábrica de aceites La central S.A de C.V. (Guadalajara, Jalisco, México).

The treatment schedule, route of administration, and dose of genistein utilized in this study were based on the findings of Rodríguez-Landa *et al.* 2009 [17] in which anxiolytic-like effects were observed in rats 12 weeks postovariectomy in the light/dark test. The treatment schedule, route of administration, and dose of tamoxifen utilized in this study were based on the findings of Walf and Frye, 2005 [24], and Gutiérrez-García *et al.* 2009 [30]. These authors found that a single injection of tamoxifen (10.0 mg/kg, s.c.) blocked the anxiolytic-like effects produced by a single injection of estrogen receptor- $\beta$  selective modulators, and treatment with tamoxifen (1.0 mg/kg, s.c.) for 6 consecutive days reduced the anxiolytic-like effects produced by estrogen receptor- $\beta$  stimulation after a single injection of testosterone. Therefore, we adjusted the schedule and doses of tamoxifen to use intermediate doses compared with the aforementioned studies.

### 2.4. Behavioral Tests

#### 2.4.1. Light/Dark Test

In the present study, the light/dark test described by Zuluaga *et al.* 2005 [31] was used. The dimensions of the apparatus were 80  $\times$  40  $\times$  40 cm. The box was further divided into two equal chambers (40  $\times$  40  $\times$  40 cm) by a barrier that had a doorway (10  $\times$  10 cm) that allowed the rats to cross freely from one chamber to the other. The dark compartment was not illuminated, whereas the light compartment was illuminated by a 40 W white light. A

video camera (Sony, DCR-SR42, 40× optical zoom, Carl Zeiss lens) was installed above the illuminated compartment to record the rat activity in the box. Later, two independent observers measured the behavioral variables until reaching a coincidence higher than 95% in measurement.

On the test day, the rats were brought to the experimental room at 6:00 PM (which was the beginning of the dark phase) and left for 1 h to acclimatize them to the novel surroundings. The light/dark test was initiated at 7:00 PM. After this time period, the rats were individually placed in the middle of the dark compartment facing the doorway, and behavioral activity was measured for 5 min. The evaluated variables were 1) frequency of entries into the light compartment (*i.e.*, total number of entries into the light compartment); 2) latency to the first entry into the light compartment (*i.e.*, the time taken after initial placement of the rat into the dark compartment until it crossed completely into the light compartment); 3) time spent in the light compartment (*i.e.*, the total time spent in the light compartment); and 4) the frequency, latency, and time spent exploring the light compartment (*i.e.*, exploration was assumed when the rat was leaning into the light compartment until its head and half of its body were in the light compartment without completely crossing into the light compartment). These variables were selected because they had been previously shown to provide a reliable measure of experimental anxiety [17, 31,32]. After the light/dark test, the rat was immediately subjected to the open field test.

#### 2.4.2. Open Field Test

In this study, general motor activity was evaluated to discard the possibility of hypoactivity, hyperactivity, or no changes in activity associated with the treatments, which could otherwise interfere with the interpretation of behavioral activity in the light/dark test [17,31]. No other measures (e.g., total number of central entries in the open field test, grooming, or rearing) were evaluated. To evaluate the effect of the treatments on spontaneous motor activity, the rats were individually subjected to a 5 mins period in the open field test. An opaque Plexiglas cage (44 × 33 cm) with walls 20 cm high was used. The floor was divided into 12 squares (11 × 11 cm). A video camera (Sony, DCR-SR42, 40× optical zoom, Carl Zeiss lens) was installed above the cage to record the activity of the rat. Later, two independent observers measured the behavioral variables until reaching a coincidence higher than 95% in measurement.

At the beginning of the test, the rat was gently placed in one of the corners of the cage. The dependent variable was the number of squares crossed by the rat (*i.e.*, crossings). A crossing was assuming when the animal passed

from one square to another with its rear legs.

After each test session, the light/dark test and open field test cage were carefully cleaned with a cleaning solution (30% ethanol) to remove the scent of the previously evaluated rat, which could otherwise modify the spontaneous behavior of the subsequent rat [33].

### 2.5. Statistical Analysis

The data from the light/dark test and open field test were analyzed using one-way analysis of variance (ANOVA) with independent groups and the Student-Newman-Keuls *post hoc* test when the *p* values reached  $\leq 0.05$ . Results are expressed as mean  $\pm$  standard error.

## 3. Results

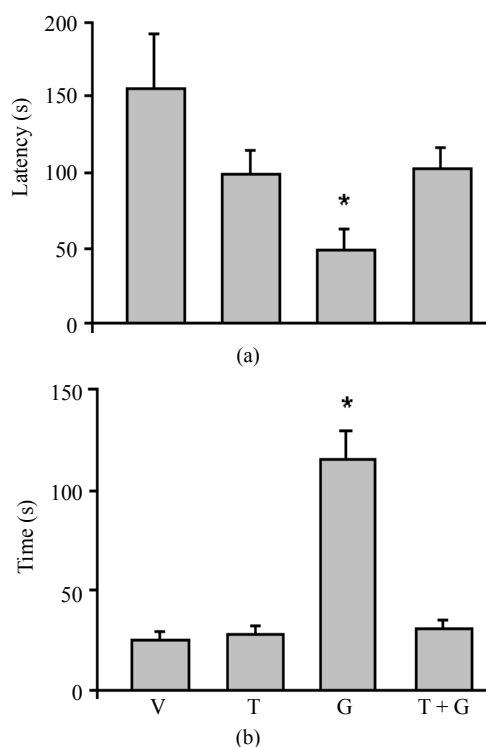
### 3.1. Light/Dark Test

The template is Frequency, latency, and time spent in the light compartment. The one-way ANOVA failed to show significant differences ( $F_{3,28} = 0.675$ ,  $p = 0.57$ ) in the frequency of entries into the light compartment among the different treatments (vehicle,  $3.00 \pm 0.46$ ; tamoxifen,  $2.75 \pm 0.59$ ; genistein,  $3.62 \pm 0.75$ ; tamoxifen + genistein,  $2.50 \pm 0.51$ ).

The one-way ANOVA showed significant differences ( $F_{3,28} = 4.260$ ,  $p < 0.013$ ) in the latency to the first entry into the light compartment. The *post hoc* test revealed that genistein treatment significantly ( $p < 0.05$ ) reduced the latency to the first entry into the light compartment compared with the control group, an effect blocked by tamoxifen pretreatment (**Figure 1(a)**). A tendency toward a reduction in the latency to the first entry into the light compartment was detected in the tamoxifen group, but no significant differences were found compared with the control group, and the differences were only attained compared with the genistein-treated group.

The analysis of the time spent in the light compartment revealed significant differences ( $F_{3,28} = 33.633$ ,  $p < 0.001$ ) among treatments. The *post hoc* test showed that rats treated with genistein spent significantly ( $p < 0.05$ ) more time in the light compartment compared with the control group, an effect blocked by tamoxifen pretreatment (**Figure 1(b)**).

Frequency, latency, and time spent exploring the light compartment. The one-way ANOVA showed significant differences ( $F_{3,28} = 13.250$ ,  $p < 0.001$ ) in the frequency exploring the light compartment among the different treatments. The *post hoc* test revealed that genistein significantly ( $p < 0.05$ ) increased the frequency exploring the light compartment compared with the control group, an effect blocked by tamoxifen pretreatment (**Figure 2(a)**).



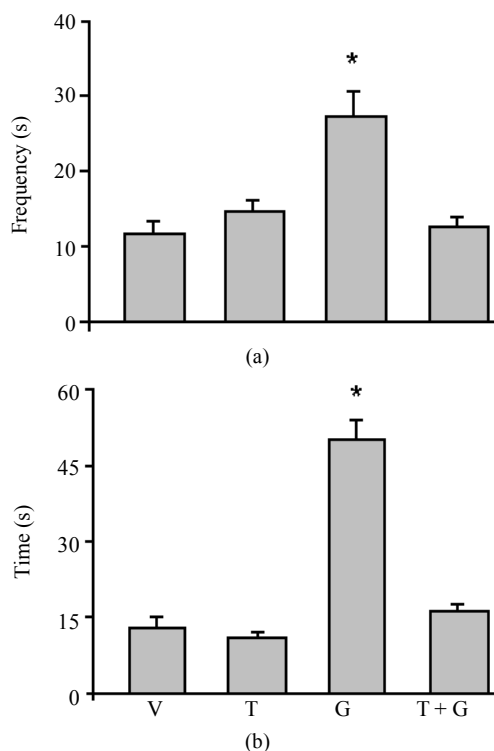
**Figure 1.** Latency to first entry into and time spent in the light compartment in the light/dark test. Genistein reduced the latency to the first entry into the light compartment (a) and increased the total time spent in this compartment (b) compared with all experimental groups. Both effects produced by genistein were blocked by pretreatment with tamoxifen. V: vehicle group; T: tamoxifen-treated group; G: genistein-treated group; T + G: tamoxifen + genistein-treated group. \*  $p < 0.05$ , compared with all experimental groups in the graphic (Student-Newman-Keuls *post hoc* test).

No significant differences ( $F_{3,28} = 3.452$ ,  $p = 0.151$ ) were detected in the latency to explore the light compartment among the different treatments (vehicle,  $10.33 \pm 1.22$  s; tamoxifen,  $11.61 \pm 0.73$  s; genistein,  $14.82 \pm 3.27$  s; tamoxifen + genistein,  $8.87 \pm 0.85$  s).

Finally, the statistical analysis revealed significant differences ( $F_{3,28} = 78.078$ ,  $p < 0.001$ ) in the time spent exploring the light compartment among the different treatments. The *post hoc* test showed that rats treated with genistein spent significantly ( $p < 0.05$ ) more time exploring the light compartment compared with the control group, an effect blocked by tamoxifen pretreatment (Figure 2(b)).

### 3.2. Open Field Test

The one-way ANOVA failed to show significant differences ( $F_{3,28} = 5.854$ ,  $p = 0.074$ ) in crossings among the different treatments, in this test all experimental groups had a similar crossing during the test session (Table 1).



**Figure 2.** Frequency and time spent exploring the light compartment in the light/dark test. The rats treated with genistein increased the frequency (a) and time spent (b) exploring the light compartment compared with all experimental groups. Both effects were blocked by pretreatment with tamoxifen. V: vehicle group; T: tamoxifen-treated group; G: genistein-treated group; T + G: tamoxifen + genistein-treated group. \*  $p < 0.05$ , compared with all groups in the graphic (Student-Newman-Keuls *post hoc* test).

**Table 1.** Effect of vehicle, genistein, tamoxifen or combination of treatments on crossing in the open field test.

Treatment	Crossing (n)
Vehicle + Vehicle	$39.37 \pm 2.07$
Tamoxifen + Vehicle	$33.62 \pm 3.06$
Vehicle + Genistein	$44.75 \pm 3.83$
Tamoxifen + Genistein	$37.01 \pm 2.39$

No significant differences were found between groups.

## 4. Discussion

In this study, the participation of estrogen receptor- $\beta$  in the anxiolytic-like effect of the phytoestrogen genistein in rats with 12-week postovariectomy in the light/dark test was explored. The main results can be summarized as the following. In the light/dark test, genistein significantly reduced the latency to the first entry into the light compartment and increased the time spent in and exploration of this compartment, supporting previous reports. Pretreatment with tamoxifen blocked the anxi-

lytic-like effects of genistein in the light/dark test in rats subjected to an experimental model of surgical post-menopause. In the open field test, no significant changes in crossings associated with the treatments were found. Altogether, these findings indicate that estrogen receptor- $\beta$  is involved in the anxiolytic-like effects produced by genistein in rats with 12-week postovariectomy in the light/dark test.

The light/dark test has been useful for the screening of substances with anxiolytic or anxiogenic potential [18, 34,35], including ovarian hormones, such as estradiol and progesterone [36,37]. In this test, an “anxious” animal exhibits an increase in the latency to the first entry into the light compartment and reduces the time spent in this compartment [18,38,39]. In contrast, animals treated with anxiolytic drugs (e.g., diazepam, alprazolam, and buspirone) spend more time in the light compartment [35,38,40], indicating an anxiolytic-like effect at experimental level. In the present study, the rats treated with phytoestrogen genistein exhibited a short latency to the first entry into the light compartment and spent more time in it compared with the control group, indicating an anxiolytic-like effect. Furthermore, genistein administration increased the frequency and time spent in exploration toward the light compartment, which is considered an additional indicator of an anxiolytic-like effect in the light/dark test, considering that diazepam and other compounds with anxiolytic effects exert similar effects [17,41]. Interestingly, the anxiolytic-like effect of genistein was blocked by pretreatment with tamoxifen, a nonspecific estrogen receptor- $\beta$  antagonist [42,43]. These data support the hypothesis that genistein exerts agonistic effects at estrogen receptor- $\beta$  [21,22,42] producing anxiolytic-like effects as occur with other positive modulators of estrogen receptor- $\beta$  at the experimental level [24]. Additionally, present results further indicate that estrogen receptor- $\beta$  would participate in the expression of anxiety-like behavior associated to low concentration of ovarian hormones produced by ovariectomy.

It is necessary point out that tamoxifen is not a pure estrogen receptor antagonist. At some dosages, tamoxifen may have agonist actions by interacting with estrogen receptor- $\alpha$ , whereas the antagonistic properties may be attributable to actions at estrogen receptor- $\beta$  [44], which occurs with others antagonists of this receptor, such as raloxifene [42,43]. In our study, no significant effects were found on the variables evaluated in the light/dark test in the tamoxifen-vehicle-treated group, discarding the possible nonspecific effects on estrogen receptor  $\alpha$  or  $\beta$  produced by the tamoxifen dose used. Altogether, our data suggest that the tamoxifen dose used in the present study acted as an estrogen receptor- $\beta$  antagonist as reported by other authors [24,30].

Finally, in the light/dark test, detecting false anxiolytic-like effects is possible when drugs increase general motor activity [18,38]. In this study, the rats treated with genistein, tamoxifen or combination of treatments did not exhibit an increase in general motor activity in the open field test, reflected by the number of crossings. Therefore, the effects produced by genistein in the light/dark test indicate an anxiolytic-like effect at the experimental level, as occur with clinically effective anxiolytic drugs, such as diazepam, which increase the time spent in the white compartment in the light/dark test, without producing significant changes in locomotion in the open field test [31, 45,46].

In conclusion, the results of this study provide a modest evidence that estrogen receptor- $\beta$  participates in the anxiolytic-like effect of the phytoestrogen genistein in the light/dark test in rats with a long-term absence of ovarian hormones. These data support the hypothesis that phytoestrogens interact with estrogen receptor- $\beta$  and produce similar effects as estrogens on mood, which could be considered in future investigations that focus on finding new therapeutic alternatives to ameliorate anxiety or depression associated with low concentrations of ovarian hormones produced by surgical or natural menopause. Further studies are also necessary to explore the possible side effects associated with long-term administration of this phytoestrogen, which occurs with other estrogenic compounds [47-50].

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