ORIGINAL ARTICLE

# Vitamin $D_3$ application attenuates anxiety-like profile and increases 25-OH-$VD_3$ levels in the serum blood of the middle-aged female rats at 12 weeks after ovariectomy

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*Submitted: 2018-08-18 Accepted: 2018-10-10 Published online: 2018-10-30*

*Key words:* **vitamin $D_3$; anxiety; behavior; estradiol; middle age; female rats**

Act Nerv Super Rediviva 2018; **60**(2): 55–66 ANSR600218A04 

## Abstract

**OBJECTIVES:** The present study was examined anxiolytic-like effects of Vitamin $D_3$ application at several doses individually alone or in a combination with low dose of 17β-estradiol in the middle-aged female rats at 12 weeks after ovariectomy.
**MATERIAL AND METHODS:** Vitamin $D_3$ (as cholecalciferol at doses of 1.0, 2.5 or 5.0 mg/kg/day, s.c.) was administered to the middle-aged ovariectomized (OVX) rats and OVX rats treated with low dose of 17β-estradiol (17β-$E_2$, 0.5 μg/rat, s.c.) after long-term ovariectomy. Anxiety-related state was tested in the elevated plus maze (EPM), light-dark test (LDT), and behavioral reactivity was registered in the open field test (OFT). Moreover, 25-hydroxyvitamin $D_3$ levels in the blood serum of the middle-aged OVX rats treated with cholecalciferol or cholecalciferol plus 17β-$E_2$ were measured.
**RESULTS:** Vitamin $D_3$ at all doses failed to modify anxiety-like behavior in the EPM and LDT test in the middle-aged intact females ($p<0.05$). The treatment with cholecalciferol at dose of 5.0 mg/kg/day induced anxiolytic-like effect in the EPM and LDT and elevated 25-OH-$VD_3$ levels in the blood of the middle-aged OVX rats ($p<0.05$). Vitamin $D_3$ at dose of 5.0 mg/kg/day plus 17β-$E_2$ more markedly produced anxiolytic-like effect in the middle-aged OVX rats ($p<0.05$). On the contrary, cholecalciferol at doses of 2.5 and 5.0 mg/kg/day failed to change anxiety-like profile of the middle-aged OVX rats in the EPM and LDT tests as compared to the control group ($p>0.05$).
**CONCLUSION:** The current findings suggest that following long-term ovariectomy in the middle-aged female rats, cholecalciferol at dose of 5.0 mg/kg/day administered alone resulted in decrease of anxiety-like behavior in the EPM and LDT. Moreover, cholecalciferol at dose of 5.0 mg/kg/day in a combination with 17β-$E_2$ induced synergic anxiolytic-like effect in the EPM and LDT tests in the OVX middle-aged rats.

**Abbreviations:**
EPM, elevated plus maze; HRT, hormonal replacement therapy; LDT, light-dark test; OFT, open field test; OVX, ovariectomized; VD, vitamin D; VDR, Vitamin D receptor

## Introduction

Women experience markedly greater prevalence of anxiety disorders than men, including generalized anxiety disorder, panic disorder, and specific phobias (Castanho *et al* 2014; Terauchi *et al* 2012). Anxiety disorders among women often precipitate or worsen at times of hormonal fluctuation, including puberty, the premenstruum, pregnancy or postpartum, and the menopausal transition (Castanho *et al* 2014). Women's transition into reproductive senes.c.ence is marked by reductions in ovarian function and output, referred to as menopause (Soares & Maki 2010). This stage is characterized by a dramatic development of affective-related disorders and different psychoemotional pathologies (Maki *et al* 2010). For women, data suggest that estrogens are strongly implicated in the regulation of mood and behavior, as well as in the pathophysiology of mood disorders (Arevalo *et al* 2015; Sherwin 1996; Walf & Frye 2006). These data are particularly important because the population continues to live longer, while the age of menopause remains unchanged (Soares 2014; Walf & Frye 2006). This extension of the lifespan means that many women will live almost half of their lives in a postmenopausal state, characterized by low estrogen levels that could possibly increase their risk for mood disorders (Soares 2014).

A strategy to alleviate the mood disorders associated with menopause is hormonal replacement therapy (HRT) (Soares 2013). However, controversial results related to the effectiveness of such treatment have been frequently reported (Soares *et al* 2001). These discrepancies could be associated to various factors, one of them being the time when estrogen restitution is initiated after the beginning of menopause (Pae *et al* 2009; Vedder *et al* 2014). Menopausal women are now choosing to take alternative and complementary therapies marketed as «natural» treatments that offer the positive health effects of estrogens without the unwanted side effects (Peng *et al* 2016; Scheid *et al* 2010). Among other nutraceuticals, one of such «natural» substances for treatment of affective-related diseases could be vitamin D (VD) (Genaro *et al* 2007; Studd & Nappi 2012).

VD is a fat soluble steroid compound performing a variety of functions in the human body (Groves *et al* 2004; Holick *et al* 1977). The active form of VD, produced in the kidneys, has a systemic effect. The vitamin produced by other cells performs within the cells or locally (Christakos *et al* 1996). Such effects are the so-called non-classic functions of VD, such as effect upon proliferation, differentiation, or apoptosis (Mizwicki & Norman 2009). Both, the classical functions, i.e., effect upon calcium-phosphate management and the non-classical ones are imposed by the nuclear receptor (VDR), regulating directly the gene expression (Cui *et al* 2007; De Luca 2014). Vitamin D receptor (VDR) belongs to the nuclear receptors, activated by a ligand and performing as transcription factors (De Luca 2014). VDR are present in most tissues and cells in the body, and within the brain show some specificity to the prefrontal cortex, hippocampus, cingulate gyrus, thalamus, hypothalamus and substantia nigra (Eyles *et al* 2005,2013,2014). This is of relevance as many of those brain regions have been implicated in the physiology of affective-related disorders (Drevets *et al* 2008).

Estrogen deficiency effects on affective-related behavior are restricted to certain periods of age after ovary removal (de Chaves *et al* 2011; Estrada-Camarena *et al* 2017). Preclinical data suggest that oneset age of menopause can be important to obtain behavioral positive or negative results with HRT alone or in a combination with some existing psychotropic drugs (Nelly *et al* 2016). We have previously demonstrated that chronic administration of Vitamin $D_3$ at doses of 1.0 and 2.5 mg/kg, subcutaneously, S.C., once daily, for 14 days had a marked anxiolytic-like effect in the adult (3 months) female rats after long-term estrogen deficiency (Fedotova *et al* 2017). On basis of the above-mentioned data of literature, we assumed that anxiolytic-like effects of Vitamin $D_3$ in the adult (3 months) OVX rats after 12 weeks post-ovariectomy can be differed from its effects in the middle-aged (12–14 months) OVX rats. Thus, it is a great interest to evaluate the effects of repeated cholecalciferol administration on anxiety-related behavior in the middle-aged female rats with long-term estrogen deficiency.

The aim of the present study was to determine if repeated systemic treatment with Vitamin $D_3$ affected on anxiety-like behavior in the middle-aged female rats after long-term ovariectomy. Another goal of this work is to clarify whether effects of repeated treatment of Vitamin $D_3$ alone or plus 17β-estradiol (17β-$E_2$) on the anxiety-like behavior of the middle-aged female rats after 12 weeks post-ovariectomy period could differ from its effects in the adult female rats after similar post-ovariectomy period.

## Material and methods

*Animals*

Female Wistar line albino rats (16–18 months old) from the special biocollection from Koltushi (St. Petersburg, Russia) weighing 230–250 g each, were used in the present study. All rats were allocated in groups and were allowed to accommodate for one week in the animal house at I.P. Pavlov Institute of Physiology, of the Russian Academy of S.c.iences, before subjecting them to behavioral testing and pharmacological treatments. They were provided with a standard pellet diet and were given water ad libitum. The animals were kept at a temperature of 23±2 °C and a 12 h light/dark cycle as well as a constant relative humidity (50% ± 10%) during all experimental sessions. throughout the experimental period. Total number of animals used in this study was 150 at the beginning of the behavioral experiments. The present study was approved by the Ethical Committee

for Animal Research, I.P. Pavlov Institute of Physiology of the Russian Academy of S.c.iences. All experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals, published by the National Institute of Health (National Research Council, publication No. 85–23, revised in 1996, and the Animal Welfare Assurance Renewal for the I.P. Pavlov Institute of Physiology, approved by the S.c.ientific Research Committee of the Institute (protocol 1095/1 from June 25, 2012).

*Surgery*

Long-term ovariectomy surgery was performed as previously described (Bosee & Di Paolo 1995). Briefly, old female rats were anesthetized with ketamine (70 mg/kg b.w.) mixed with xylazine (10 mg/kg b.w.). To avoid inflammation, the rats were administered with meloxicam (1 mg/kg b.w.). The fallopian tube was crushed and the ovary was removed by cutting. The effectiveness of long-term ovariectomy or 17β-estradiol (17β-$E_2$) application was assessed by vaginal smears. The old ovariectomized (OVX) females were housed in groups of five in cages separated by groups. To assure the long-term absence of estrogens, all rats after surgery were remained to the housing facilities for 12 weeks.

*Drug treatments*

17β-Estradiol, 17β-$E_2$ (E-8875, Sigma Chemical Co., St. Louis, MO, USA) at low dose of 5.0 μg/rat (Estrada-Camarena *et al* 2003,2004) and Vitamin $D_3$ as cholecalcirefol (C-9756, Sigma Chemical Co., St. Louis, MO, USA) at several doses (1.0, 2.5 or 5.0 mg/kg) (Idrus *et al* 2013) were subcutaneously (s.c.) administered once daily starting 14 days prior to the cognitive experiments. 17β-$E_2$ was dissolved in sterile sesame oil, $VD_3$ – in 95% ethanol solvent, aliquoted and stored at –80 °C. The stock of $VD_3$ was dissolved in a sterile water, resulting in a solution of cholecalciferol with 2% ethanol. All drug solutions were freshly prepared before each behavioral testing. 17β-$E_2$ and cholecalcirefol were injected in a volume of 0.1 mL. The middle-aged OVX females were 15.5–17.5 months old at the onset for drug treatments.

*Experimental groups*

In our previous studies (data are not shown), we did not found any differences between control groups of middle-aged intact females with oil solvent and solvent for cholecalciferol, we used only one control middle-aged intact (sham-operated) group with oil solvent.

Twelve weeks after ovariectomy, the middle-aged OVX female rats were randomly assigned to each of the experimental groups and subjected to the different treatments. All middle-aged female OVX and intact rats were divided into 12 groups (n=8 per group) for each behavioral tests. The first group consisted of middle-aged intact (sham-operated) female rats (control) daily treated with oil solvent (control + solvent). The three other groups were of middle-aged intact (sham-operated) female rats which received cholecalciferol at a daily dose of 1.0 mg/kg S.C. (intact rats + cholecalciferol 1.0 mg/kg), cholecalciferol at a daily dose of 2.5 mg/kg S.C. (intact rats + cholecalciferol 2.5 mg/kg) or cholecalciferol at a daily dose of 5.0 mg/kg S.C. (intact rats + cholecalciferol 5.0 mg/kg). The next two groups were of middle-aged OVX female rats received the oil solvent daily (OVX + solvent) and middle-aged OVX rats treated with 17β-$E_2$ at a daily dose of 0.5 μg/rat, s.c. (OVX + 17β-$E_2$). The other groups consisted of the middle-aged OVX female rats treated with cholecalciferol at a dose of 1.0 mg/kg (OVX rats + cholecalciferol 1.0 mg/kg), middle-aged OVX female rats treated with cholecalciferol at a dose of 2.5 mg/kg (OVX rats + cholecalciferol 2.5 mg/kg), middle-aged OVX female rats treated with cholecalciferol at a dose of 5.0 mg/kg (OVX rats + cholecalciferol 5.0 mg/kg), middle-aged OVX female rats treated with cholecalciferol at a dose of 1.0 mg/kg plus 17β-$E_2$ (OVX rats + cholecalciferol 1.0 mg/kg + 17β-$E_2$), middle-aged OVX female rats treated with cholecalciferol at dose of 2.5 mg/kg plus 17β-$E_2$ (OVX rats + cholecalciferol 2.5 mg/kg + 17β-$E_2$), and middle-aged OVX female rats treated with cholecalciferol at a dose of 5.0 mg/kg plus 17β-$E_2$ (OVX rats + cholecalciferol 5.0 mg/kg + 17β-$E_2$).

In fact, after induction of the experimental model of estrogen deficiency, the middle-aged OVX rats were left to recover for 12 weeks. After that time, the middle-aged OVX female rats began daily injections for 14 days with either cholecalciferol, 17β-$E_2$ or oil solvent. One hour after the last injection, testing in elevated plus maze (EPM), in the light-dark test (LDT) and the open field test (OFT) was carried out as described below. During all behavioral tests, the experimental groups of the middle-aged OVX rats were also treated with cholecalciferol, 17β-$E_2$ or solvent.

*Behavioral tests*

**Elevated plus maze test.** To investigate the changes in anxiety-like behavior, control intact (sham-operated) rats and all experimental groups of OVX female rats with long-term absence of estrogen were subjected to the elevated plus maze test (EPM) (Pellow & File 1986). EPM is a widely used test of anxiety-like behavior and was used to assess an anxiety-like behavioral responses (Pellow *et al* 1985). This test is sensitive to putative anxiogenic-like and anxiolytic-like drugs (Pellow & File 1986).The apparatus was made of grey Plexiglas and consisted of four arms (50 cm long and 10 cm wide); two arms had 40-cm-high dark walls (closed arms), and two arms had 0.5-cm-high ledges (open arms). In the center of the arms of EPM located cross-wise there was an open area in the size of 10 × 10 cm. The floor of the apparatus was 50 cm high. The experimental room was lit by a 60 Watt bulb placed 1.75 m above the central square of the maze (22 lx in the maze central square). For testing, rats were placed individually into the center of

the maze facing a closed arm and removed after a 5-min period. The number of entrances and the time spent into the open or closed arms were registered during time of testing. A video camera was installed above the cage to record the activity of the rats. Two independent observers measured the behavioral variables. After each test session, the EPM apparatus was carefully cleaned and deodorized with the cleaning solution.

**Light/dark test.** The light/dark test (LDT) was used to test unconditioned anxiety and exploratory behavior. It is based on the natural aversion of rodents to bright light in novel environments (Edinger & Frye 2007; Pan & Chen 2006). The apparatus was consisted of a Plexiglas box with two equal compartments (30×40×40 cm), one of which with white walls and floor and illuminated by a 60 Watt light from above, while the other of the box was painted black and had a lid so it was not illuminated. The time spent and the number of entrances in the illuminated compartment were recorded for 5 min (Walf & Frye 2005). Increased time in the light side is indicative of anti-anxiety behavior. A video camera was installed above the cage to record the activity of the rats. Two independent observers measured the behavioral variables. After each test session, the LDT apparatus was carefully cleaned and deodorized with the cleaning solution.

**Open field test.** To investigate the changes in behavioral reactivity, all experimental groups of offspring were submitted to a 5-min period to the open field test (OFT) as described previously (Fedotova *et al* 2012). Two independent observers registered the behavioral variables. A video camera was installed above the cage to record the activity of the rats. After each test session, the OFT apparatus was carefully cleaned and deodorized with the cleaning solution.

*Vitamin $D_3$ status*

Approximately 5 ml of blood samples were drawn from animals anesthetized with ketamine (5.0–10 mg/kg, i.m.). After centrifugation, serum samples were frozen at –20 °C until analysis. Afterthat, serum samples were used for the measurement of Rat 25-hydroxyvitamin $D_3$ (25-OH-$VD_3$) levels using a commercially available ELISA kit (CSB-E08098r, Cusabio Biotech Co., Ltd, Wuhan, P.R. China). Technical variability was low with coefficients of variation of <10% intra-assay and <15% inter-assay. Detection range is 20–100 µg/L. The sensitivity of the ELISA was 5.0 µg/L.

*Statistical analysis*

All values were expressed as mean ± S.E.M. Comparisons between values were performed using two-way ANOVA test with between subject factors for hormone state (middle-aged OVX or OVX plus 17β-$E_2$) and drug treatment (oil solvent or cholecalciferol) followed by Dunnett's test for multiple comparisons post-hoc test. Statistical analysis was performed using SPSS version 11.5 (SPSS Inc., Chicago, IL., USA).

## Results

*Vitamin $D_3$ modulates anxiety-like behavior of middle-aged OVX and OVX rats treated with 17β-estradiol in the elevated plus maze*

A two-way ANOVA revealed significant differences in the time spent into the open arms between hormone conditions ([$F(5,44) = 9.47$, $p<0.01$]), between drug treatments ([$F(5,44) = 15.24$, $p<0.05$]), and an interaction between hormone condition and treatments ([$F(5,44) = 12.83$, $p<0.05$]) in the middle-aged OVX rats with long-term estrogen deficiency-induced anxiety. The post-hoc test revealed differences among the groups for anxiety-like behavior in the EPM ($p<0.05$).

The intact middle-aged rats treated with cholecalciferol at all tested doses failed to demonstrate any modifications in the time spent into the open arms as compared to the control rats (Figure 1a, $p>0.05$). Long-term ovariectomy in the middle-aged female rats resulted in a significant decrease of the time spent in the open arms as compared to the control females (Figure 1a, $p<0.05$). The 17β-$E_2$ supplementation (0.5 µg/kg, s.c.) caused an increase in the time spent in the open arms in the middle-aged OVX rats as compared to the middle-aged OVX rats administered solvent (Figure 1a, $p<0.05$). Although the values of these parameters in the middle-aged OVX rats treated with 17β-$E_2$ were higher than that of the middle-aged OVX rats given with solvent, they did not reach the values of control sham-operated rats (Figure 1a).

The *post-hoc* test failed to reveal significant differences among the groups of the middle-aged OVX rats treated with cholecalciferol at a dose of 1.0 mg/kg or 2.5 mg/kg for anxiety-like behavior in the EPM as compared to the control group (Figure 1a, $p>0.05$). On the contrary, cholecalciferol treatment (5.0 mg/kg, s.c.) significantly increased the time spent in the open arms of the middle-aged OVX rats as compared to the middle-aged OVX and control rats given solvent (Figure 1a, $p<0.05$). Administration of cholecalciferol at dose of 5.0 mg/kg in combination with 17β-$E_2$ more significantly increased the time spent in the open arms for the middle-aged OVX rats as compared to the middle-aged OVX females treated with oil solvent or 17β-$E_2$ (Figure 1a, $p<0.05$). The time spent in the open arms for middle-aged OVX rats administered cholecalciferol at doses of 1.0 mg/kg or 2.5 mg/kg in combination with 17β-$E_2$ was significantly greater than that of the middle-aged OVX rats given solvent, but did not reach the value of middle-aged control rats (Figure 1a, $p<0.05$). Moreover, the values of time spent in the open arms of middle-aged OVX rats administered with cholecalciferol at doses of 1.0 mg/kg or 2.5 mg/kg in combination with 17β-$E_2$ were similar to the values for OVX rats treated with 17β-$E_2$.

Similarly, significant differences in the number of entries into the open arms were found between hormone conditions ($F(5,44) = 7.88$, $p<0.01$), between drug

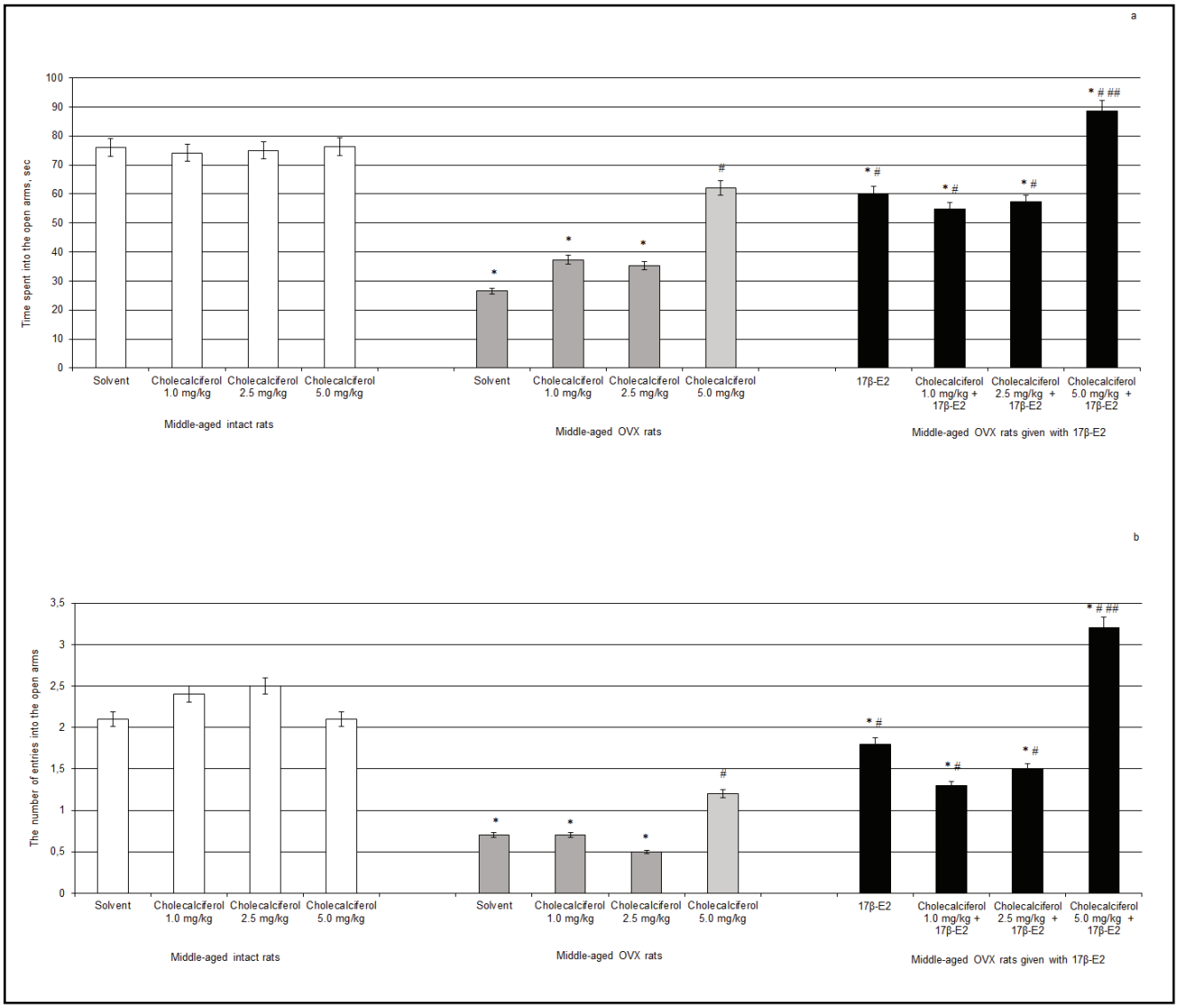


**Fig. 1.** Vitamin $D_3$ modulates anxiety-like behavior of the middle-aged ovariectomized (OVX) rats following long-term estrogen deficiency in the elevated plus maze. (a) – time spent into the open arms, sec; (b) – the number of entries into the open arms. *$p$<0.05 versus the control group, **$p$<0.05 versus to the old OVX rats treated with solvent, ## – $p$<0.05 versus the old OVX rats treated with 17β-estradiol (17β-$E_2$). The data are expressed as mean ± SD; n=8 in each group.

treatments (F(5,44) = 11.02, $p$<0.05), and an interaction between hormone condition and treatments (F(5,44) = 9.47, $p$<0.01) in the middle-aged OVX rats. The post-hoc test revealed differences among the groups for anxiety-like behavior in the EPM ($p$<0.05).

The middle-aged intact rats treated with cholecalciferol at doses of 1.0 mg/kg, 2.5 mg/kg or 5.0 mg/kg showed no alteration in the number of entries into the open arms as compared to the middle-aged control rats (Figure 1b, $p$>0.05). The middle-aged intact rats administered with cholecalciferol at a dose of 5.0 mg/kg showed a significant increase with respect to the number of entries into the open arms as compared to the control rats (Figure 1b, $p$<0.05). The middle-aged OVX rats given solvent displayed a significant decrease in the number of entries into the open arms as compared to the middle-aged control rats (Figure 1b, $p$<0.05). Administration of 17β-$E_2$ to the middle-aged OVX rats increased the number of entries into the open arms as compared to the middle-aged OVX rats treated with solvent (Figure 1b, $p$<0.05). Although the values of these parameters in the middle-aged OVX rats treated with 17β-$E_2$ were higher than those of the OVX rats given with solvent, they did not reach the values of control rats (Figure 1b, $p$<0.05).

The number of entries into the open arms was significantly higher when the middle-aged OVX rats treated with cholecalciferol at dose of 5.0 mg/kg were compared to the middle-aged OVX rats given with solvent (Figure 1b, $p$<0.05). Cholecalciferol at doses of 1.0 mg/kg or 2.5 mg/kg failed to change the number of entries into the open arms in the middle-aged OVX rats as compared to the OVX solvent-receiving rats (Figure 1b, $p$<0.05). Administration of dose of 5.0 mg/kg in combination

with 17β-$E_2$ to the middle-aged OVX rats more significantly increased the number of entries into the open arms as compared to the middle-aged OVX rats treated with solvent or 17β-$E_2$ (Figure 1b, $p<0.05$). The number of entries into the open arms of middle-aged OVX rats administered cholecalciferol at doses of 1.0 mg/kg or 2.5 mg/kg in combination with 17β-$E_2$ was lower than that of the middle-aged OVX rats given solvent, but this did not reach the value of the middle-aged control rats (Figure 1b, $p<0.05$). Furthermore, the value for the number of entries into the open arms of the middle-aged OVX rats administered cholecalciferol at doses of 1.0 mg/kg or 2.5 mg/kg plus 17β-$E_2$ was similar to the value for middle-aged OVX rats treated with 17β-$E_2$.

*Vitamin $D_3$ reduces anxiety-like behavior of middle-aged OVX and OVX rats treated with 17β-estradiol in the light-dark test*

The two-way ANOVA showed significant differences in the time spent and number of entries in the light compartment between hormone conditions ($F(5,44) = 19.22$, $p<0.05$) and ($F(5,44) = 9.56$, $p<0.001$), between drug treatments ($F(5,44) = 9.88$, $p<0.01$) and ($F(5,32) = 7.26$, $p<0.05$), and an interaction between hormone condition and treatments ($F(5,44) = 11.52$, $p<0.01$) and ($F(5,44) = 16.75$, $p<0.05$) in the middle-aged OVX rats. The *post-hoc* test revealed differences among the groups for anxiety-like behavior in the LDT ($p<0.05$).

The middle-aged intact rats treated with cholecalciferol at doses of 1.0 mg/kg, 2.5 mg/kg and 5.0 mg/kg failed to modify the time spent and number of entries in the light box as compared to the middle-aged control rats (Figure 2a,b, $p>0.05$). The middle-aged OVX rats given solvent displayed a significant decrease of the time spent and number of entries in the light box as compared to the middle-aged control rats (Figure 2a,b, $p<0.05$). Administration of 17β-$E_2$ to the middle-aged OVX rats elevated the time spent and number of entries in the light box as compared to the middle-aged OVX rats treated with solvent (Figure 2a,b, $p<0.05$). Although the values of these parameters in the middle-aged OVX rats treated with 17β-$E_2$ were higher than those of the

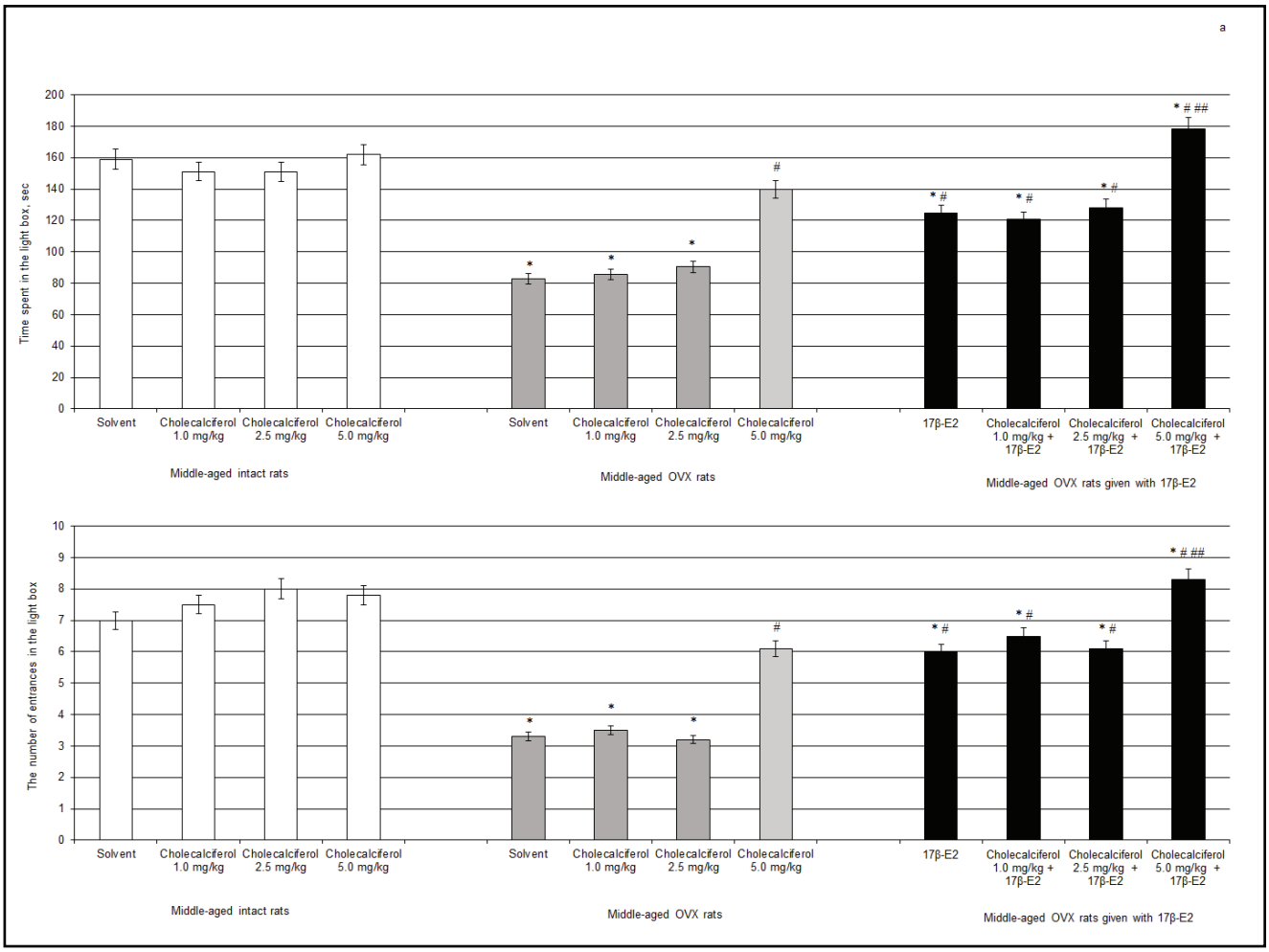


**Fig. 2.** Changes of anxiety-like behavior of the middle-aged ovariectomized (OVX) rats treated with Vitamin $D_3$ or Vitamin $D_3$ plus 17β-estradiol in the light-dark test. (a) – time spent in the light box, sec; (b) – the number of entrances in the light box. *$p<0.05$ versus the control group, **$p<0.05$ versus to the old OVX rats treated with solvent, ##$p<0.05$ versus the old OVX rats treated with 17β-estradiol (17β-$E_2$). The data are expressed as mean ± SD; n=8 in each group.

**Tab. 1.** Cholecalciferol influences on behavioral parameters of the middle-aged OVX rats following long-term estrogen deficiency in the open field test for 5 min.

| Groups | Crossing | Rearing | Grooming |
|---|---|---|---|
| Middle-aged control rats + solvent | 73.3±4.2 | 12.1±0.8 | 3.0±0.2 |
| Middle-aged intact rats + cholecalciferol 1.0 mg/kg | 68.0±2.4 | 12.0±0.5 | 3.2±0.2 |
| Middle-aged intact rats + cholecalciferol 2.5 mg/kg | 59.3±5.6 | 10.5±0.8 | 3.2±0.2 |
| Middle-aged intact rats + cholecalciferol 5.0 mg/kg | 69.5±4.2 | 11.7±0.8 | 3.5±0.2 |
| Middle-aged OVX rats + solvent (OVX/solvent rats) | 62.4±2.3 | 12.3±0.6 | 1.2±0.5* |
| Middle-aged OVX rats + 17β-$E_2$ (OVX/17β-$E_2$ rats) | 60.3±2.6 | 13.2±0.3 | 3.3±0.4# |
| Middle-aged OVX rats + cholecalciferol 1.0 mg/kg | 65.2±2.5 | 12.1±0.6 | 4.0±0.2# |
| Middle-aged OVX rats + cholecalciferol 2.5 mg/kg | 76.9±4.2 | 13.2±0.8 | 3.8±0.4# |
| Middle-aged OVX rats + cholecalciferol 5.0 mg/kg | 72.3±4.4 | 11.0±0.5 | 4.1±0.6# |
| Middle-aged OVX rats + cholecalciferol 1.0 mg/kg + 17β-$E_2$ | 62.1±2.8 | 12.6±0.6 | 0.6±0.2*### |
| Middle-aged OVX rats + cholecalciferol 2.5 mg/kg + 17β-$E_2$ | 73.2±2.4 | 10.2±0.4 | 0.5±0.2*### |
| Middle-aged OVX rats + cholecalciferol 5.0 mg/kg + 17β-$E_2$ | 65.4±5.6 | 12.5±0.8 | 0.8±0.2*### |

*p<0.05 versus the control group, **p<0.05 versus to the old OVX rats treated with solvent, ## – p<0.05 versus the old OVX rats treated with 17β-estradiol (17β-$E_2$). The data are expressed as mean ± SD; n=8 in each group.

middle-aged OVX rats, they did not reach the values of control rats. The middle-aged OVX rats treated with cholecalciferol at dose of 5.0 mg/kg showed an increase in the time spent and number of entries in the light box as compared to the middle-aged OVX rats given with solvent (Figure 2a,b, $p<0.05$). Administration of cholecalciferol at doses of 1.0 mg/kg and 2.5 mg/kg to the middle-aged OVX females failed to change the time spent and number of entries in the light box as compared to the middle-aged OVX or intact rats given solvent (Figure 2a,b, $p<0.05$). The treatment with cholecalciferol at dose of 5.0 mg/kg in combination with 17β-$E_2$ in the middle-aged OVX rats more significantly increased the time spent and number of entries in the light box as compared to the middle-aged intact control and OVX rats treated with solvent or 17β-$E_2$ (Figure 2a,b, $p<0.05$). The time spent and number of entries in the light box in the OVX rats administered cholecalciferol at doses of 1.0 mg/kg and 2.5 mg/kg in combination with 17β-$E_2$ were higher than that of the middle-aged OVX rats given solvent, but did not reach the values of middle-aged control rats (Figure 2a,b, $p<0.05$). Furthermore, the values for the time spent and number of entries in the light box of the middle-aged OVX rats administered cholecalciferol at doses of 1.0 mg/kg and 2.5 mg/kg plus 17β-$E_2$ were similar to the values for middle-aged OVX rats treated with 17β-$E_2$.

*Vitamin $D_3$ affects grooming behavior of middle-aged OVX and OVX rats treated with 17β-estradiol in the open field test*

The two-way ANOVA revealed significant differences in the grooming behavior between hormone conditions ($F(5,44) = 8.12$, $p<0.05$), between drug treatments ($F(5,44) = 12.51$, $p<0.01$), and an interaction between hormone condition and treatments ($F(5,44) = 7.16$, $p<0.01$) in the middle-aged OVX rats. The *post-hoc* test revealed differences among the groups for behavior in the OFT ($p<0.05$).

The middle-aged female rats treated with cholecalciferol at all doses failed to demonstrate any changes in the behavioral reactions in the OFT as compared to the middle-aged control rats (Table 1, $p>0.05$). The middle-aged OVX rats given with solvent demonstrated a significant decrease of grooming behavior as compared to the middle-aged control rats (Table 1, $p<0.05$). The 17β-$E_2$ supplementation produced a significant increase in grooming reactions when these rats were compared to the middle-aged OVX rats treated with solvent (Table 1, $p<0.05$). The post-hoc test failed to reveal any alterations of motor and rearing activities in the middle-aged OVX rats treated with cholecalciferol in all tested doses alone or in a combination with 17β-$E_2$ as compared to the middle-aged OVX rats given with solvent (Table 1, $p<0.05$). On the contrary, the middle-aged OVX rats treated with cholecalciferol at 1.0, 2.5 and 5.0 mg/kg showed an increase of grooming behavior as compared to the middle-aged OVX rats. The combination of cholecalciferol at these doses with 17β-$E_2$ decreased grooming behavior as compared to the middle-aged intact, OVX rats received with solvent or 17β-$E_2$ (Table1, $p<0.05$).

*Vitamin $D_3$ increased on 25-hydroxyvitamin $D_3$ levels in the blood serum of middle-aged OVX females and OVX females treated with 17β-estradiol*

A two-way ANOVA revealed significant differences in 25-hydroxyvitamin $D_3$ (25-OH-$VD_3$) levels between

hormone conditions ([F(5,44) = 11.32, $p<0.01$), between drug treatments ([F(5,44) = 7.4, $p<0.05$]), and an interaction between hormone condition and treatments ([F(5,44) = 16.33, $p<0.01$] and F(5,44) = 14.11, $p<0.05$]) in the middle-aged OVX rats with long-term estrogen deficiency. The post-hoc test revealed differences among the experimental groups for 25-OH-$VD_3$ and estradiol levels ($p<0.01$ and $p<0.05$, respectively).

The middle-aged intact rats treated with cholecalciferol at doses of 1.0, 2.5 and 5.0 mg/kg increased 25-OH-$VD_3$ levels (Figure 3, $p<0.05$).

Long-term ovariectomy in the middle-aged female rats resulted in a significant decrease of 25-OH-$VD_3$ levels in the blood as compared to the middle-aged control females (Figure 3, $p<0.05$). The 17β-$E_2$ supplementation (0.5 μg/kg, s.c.) failed to modify 25-OH-$VD_3$ levels in the blood of the middle-aged OVX rats as compared to the middle-aged OVX rats administered with solvent (Figure 3, $p>0.05$), and the value of this parameter in the middle-aged OVX/17β-$E_2$ females were lower than that of the value of middle-aged control rats.

The middle-aged OVX rats treated with cholecalciferol at doses of 2.5 and 5.0 mg/kg significantly increased 25-OH-$VD_3$ levels in the serum blood as compared to the middle-aged OVX rats treated with solvent (Figure 3, $p<0.05$). Moreover, cholecalciferol treatment at doses of 2.5 and 5.0 mg/kg in combination with 17β-$E_2$ more significantly enhanced 25-OH-$VD_3$ levels for the middle-aged OVX rats as compared to the OVX females treated with oil solvent or 17β-$E_2$ (Figure 3, $p<0.05$). However, cholecalciferol administered at a dose of 1.0 mg/kg alone or in a combination with 17β-$E_2$ into the middle-aged OVX rats failed to change 25-OH-$VD_3$ levels in the serum blood as compared to the middle-aged OVX rats treated with solvent (Figures 3, $p>0.05$).

## Discussion

In the present work, the effects of chronic cholecalciferol treatment at different doses (1.0, 2.5 and 5.0 mg/kg, S.C.) for 14 days on anxiety-like behavior in the middle-aged female rats with long-term estrogen deficiency and 17β-$E_2$ supplementation in a low dose were examined. Endogenous estrogens were removed by ovariectomy and only after 12 weeks post-ovariectomy period, these rats were used in all experiments. The results of behavioral testing for the anxiety-related effects of cholecalciferol were compared in both middle-aged OVX rats and OVX female rats treated with 17β-$E_2$. Simultaneously, the effects of cholecalciferol at similar doses on anxiety-like behavior were tested in intact female rats. For this purpose, the elevated plus maze (EPM) and light-dark test (LDT) were made in the present study. It was also investigated whether the effects of cholecalciferol at different doses were specific in the EPM and LDT, measuring its effects on the behavioral activity in the OFT of the middle-aged intact and OVX rats after long-term absence of estrogen.

Vitamin $D_3$ at all investigated doses did not produce any changes of anxiety-like behavior of the middle-aged (12–14 months) intact female rats in the EPM and LDT. In contrast to our previous study where intact rats were 3 months of age, cholecalciferol exhibited anxiolytic-like effect at a dose of 5.0 mg/kg, in the EPM and LDT. Analyzing the results from biochemical assay, dose-dependent increase of 25-OH-$VD_3$ concentrations in the serum blood of the middle-aged intact rats given with different doses of cholecalciferol were found. These results suggest that cholecalciferol-induced changes of 25-OH-$VD_3$ status in the blood serum of the middle-aged intact-ovary rats are not associated any alterations of anxiety-like profile in the EPM and

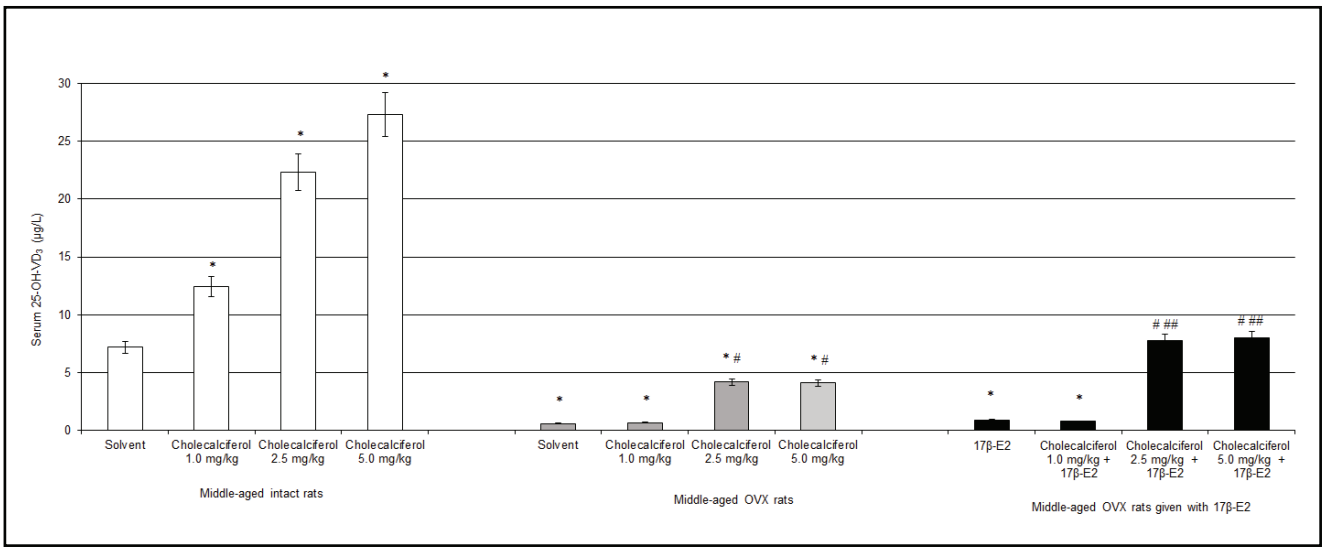


**Fig. 3.** 25-OH-$VD_3$ levels in the serum blood of the middle-aged ovariectomized (OVX) rats treated with Vitamine $D_3$ alone or in a combination with 17β-estradiol. *$p<0.05$ versus the control group, **$p<0.05$ versus to the old OVX rats treated with solvent, ##$p<0.05$ versus the old OVX rats treated with 17β-estradiol (17β-$E_2$). The data are expressed as mean ± SD; n=8 in each group.

LDT. The current study has some limitations. We did not measured the phase of ovary cycle in intact females. The next step will be assessment of cholecalciferol in different doses effects on anxiety-like behavior in the middle-aged female rats for all phases of ovary cycle. Moreover, the ovary-intact female rats of different age are also needed to evaluate the behavioral effects of cholecalciferol administered at several doses in the EPM and LDT paradigms.

The results showed that in the middle-aged OVX rats following 12 weeks of postovariectomy period, there were marked anxiety-like behavior as assessed by EPM and LDT. Although 17β-$E_2$ supplementation resulted in significant anxiolytic-like effect of the middle-aged OVX rats with long-term absence of estrogen, the 17β-$E_2$ administration was not able to completely diminish anxiety-like behavior to the level of the middle-aged control intact animals. According to these results, we conclude that middle-aged OVX rats following 12 weeks of postovariectomy period display significant anxiety-related behavior, while 17β-$E_2$ administration to the middle-aged OVX rats attenuates the estrogen deficiency-induced anxiety-like behavior to some extent. In fact, these experiments showed that the effects of 17β-$E_2$ supplementation on anxiety-like behavior did not associated with absence of its effects on 25-OH-$VD_3$ levels in the middle-aged OVX rats. The long-term effect of ovariectomy on anxiety-like behavior in female rats that were submitted in a standard behavioral tests (Okada *et al* 1997).

Cholecalciferol at dose of 5.0 mg/kg/day per se had a significant anxiolytic-like effect in the middle-aged OVX rats following long-term ovariectomy. On the contrary, cholecalciferol at doses of 1.0 and 2.5 mg/kg/day failed to induce any modifications of anxiety-like behavior in the middle-aged OVX rats with long-term absence of estrogen. Interestingly, in the present study cholecalciferol exhibited anxiolytic-like effect only at a dose of 5.0 mg/kg in the middle-aged OVX rats with long-term absence of estrogen. In contrast to our previous study, where OVX rats was 3 months old, the dose of 5.0 mg/kg, S.C. of cholecalciferol increased anxiety-like behavior. Moreover, we demonstrated anxiolytic-like effects of cholecalciferol only at doses of 1.0 and 2.5 mg/kg in the EPM and LDT (Fedotova *et al* 2017). Simultaneously, cholecalciferol treatment in all tested doses similarly increased grooming, did not change locomotor activity and rearing of the middle-aged OVX rats after long-term ovariectomy. However, its effects on the manifestation for anxiety-like behavior of these rats were completely different in the EPM and LDT. These data suggested that the different effects of cholecalciferol application at all doses in the middle-aged OVX rats with long-term absence of estrogen on anxiety-like behavior in the EPM and LDT did not associated with its effects on behavioral reactions in the OFT. The obtained data generally confirmed the anxiety-like activity of cholecalciferol in the middle-aged OVX females with long-term absence of estrogen and indicates that the effects of cholecalciferol are specific, since any alterations in motor or grooming activities were not involved in its action in the EPM and LDT tests.

ELISA assays demonstrated that administration of cholecalciferol only at doses of 2.5 and 5.0 mg/kg resulted in elevated 25-OH-$VD_3$ levels in the blood serum of the middle-aged OVX rats. Moreover, application of cholecalciferol at these doses with low dose of 17β-$E_2$ induced more profound increase of 25-OH-$VD_3$ levels in the blood serum of the middle-aged OVX rats. These data suggested that the different effects of cholecalciferol application in the middle-aged OVX rats on anxiety-like behavior in the EPM and LDT did not associated with its effects on 25-OH-$VD_3$ levels.

Administration of cholecalciferol at dose of 5.0 mg/kg/day in a combination with low dose of 17β-$E_2$ in the middle-aged OVX rats after long-term absence of estrogen exhibited synergic action and potentiated the anxiolytic-like effects of both preparations in the EPM and LDT. However, the middle-aged OVX rats after long-term absence of estrogen treated with cholecalciferol at all doses and low dose of 17β-$E_2$ demonstrated lower grooming than middle-aged OVX rats given with low dose of 17β-$E_2$ alone. The middle-aged OVX rats with 12 weeks postovariectomy period administered with cholecalciferol at doses 1.0 and 2.5 mg/kg/day in combination with 17β-$E_2$ showed similar anxiety-like profile like the OVX rats given with solvent. In fact, the effects of cholecalciferol at different doses alone or in a combination with a low dose of 17β-$E_2$ on the anxiety-like behavior of the middle-aged OVX rats after long-term absence of estrogen were specific, since effects cholecalciferol on behavioral reactions did not associated with its effects on behavioral activity of these middle-aged OVX rats in the OFT.

Thus, in the present study, it was observed that at 12 weeks postovariectomy period only a dose of 5.0 mg/kg cholecalciferol was effective to reduce anxiety-like behavior in the EPM and LDT in the middle-aged OVX rats. In contrast, at 3 months old OVX rats a lower doses of cholecalciferol (1.0 and 2.5 mg/kg) produced anxiolytic-like effect in in the EPM and LDT (Fedotova *et al* 2017). Current data suggest that the effects of cholecalciferol at tested doses on the anxiety-like profile are different from the adult (3 months) and the middle-aged (12–14 months) OVX females. Moreover, age of OVX rats significantly alters the anxiolytic-like response of treatment with cholecalciferol in OVX rats. It is possible that specific sites of action involved in the anxiolytic-like effects of cholecalciferol that also modulated by estrogens are affected by the endocrine milieu that prevails at different period for the adult and the middle-aged female rats. Moreover, after a long-time absence of ovarian fluctuations an adaptive process may contribute to a better response for cholecalciferol administration at a dose of 5.0 mg/kg in the middle-aged rats.

The role of ovarian hormones in anxiety and stress sensitivity is of great interest for women transitioning through menopause (Burger 2008; Maclennan *et al* 2004). Mood disorders during menopause could be partly due to loss of estrogen with menopause because estrogen is known to have neuroprotective effects on brain (Przybelski & Binkley 2007; Wilkins *et al* 2006). HRT may improve the symptoms of affective-related state in people or decrease the risk of developing mood disturbances in older women, but this is unclear because in some studies MHT does not stop the development of anxiety-like symptoms in elderly postmenopausal women (Rossouw *et al* 2002). The exact role of estrogen still needs to be defined. Menopause are also at higher risk of developing VD deficiency due to decreased dietary intake, less sun exposure, restricted outdoor activity and a decreased capacity to produce enough calcitriol as a result of an age related decline in hydroxylation by kidneys (Cheema *et al* 1989; Robbins *et al* 2014; Schnatz *et al* 2012). VD, a group of steroid compounds, has become of great interest due to many studies which have revealed its role far beyond bone metabolism (Kesby *et al* 2011; Stewart *et al* 2010). Through decades VD was considered a vitamin but nowadays it has emerged as an active hormone exerting its action as a trans.c.ription factor regulating the expression of numerous genes (Holick 2006; Penckofer *et al* 2010). The presence of VD receptors (VDR) outside the skeletal system, enterocytes and renal tubular cells was confirmed in many cell types including immune cells, neurons, pancreatic cells, myocytes, cardiomyocytes, endothelium cells, which stress pleiotropic activity of VD (Holick 2007). There is a great body of evidence confirming that apart from its well-known function in calcium-phosphate homeostasis, VD also exerts many non-calcemic actions in various tissues and systems (Fernandes de Abreu *et al* 2009). VD deficiency has been linked with significant complications such as cardiovascular events, depression, anxiety, cognitive disorders, obesity, metabolic syndrome, type 2 diabetes, various types of cancer, immune disorders (Fernandes de Abre *et al* 2009). According to Gaugris and co-workers (2005), the prevalence of low VD levels appears to be high in postmenopausal women. Additionally, the decline of estrogens after menopause decreases the activity of 1α-OHase, what results in lower synthesis of the active VD form (Bikle 2014). These results suggest that VD supplementation, even in higher doses, may be necessary in postmenopausal women. VD supplementation seems to be the most appropriate treatment option for the population of postmenopausal patients and has been suggested by many experts as a safe and cost-effective procedure. However, the role of VD supplementation in the prevention and treatment of comorbidities associated with menopausal consequences has not been completely established.

Adequate VD status may play a very important role in terms of appropriate brain development and function (Kalueff *et al* 2004; Garsion *et al* 2002). Therefore, adequate supply of VD in specific periods of life, including the menopausal period, seems to be of particular importance, because it may reduce the risk of CNS diseases whose treatment is difficult and which represent a heavy burden both for the affected individuals and their society (Halloran & De Luca 1980; Eyles *et al* 2003). What becomes particularly important in light of these reports is continued study of the effects of VD on CNS function aimed at establishing a recommendation of VD dietary intake, which is a key element in averting its deficiency, and making tests determining serum 25(OH)D concentration generally available in menopausal women (Vedder *et al* 2014). These points illustrate how the current state of VD treatment research is incomplete and in need of more intensive research. Working toward uncovering how the interaction between VD and estradiol changes after menopause, and the implications of these changes elsewhere in the post-menopausal woman, is necessary for providing the most complete understanding of how VD treatment alone or in a combination with 17β-estradiol supplementation may affect women's affective-related state.

The female reproductive system is composed of central regulators including the hypothalamus and the pituitary gland and peripheral organs such as the ovary, uterus, and during pregnancy the placenta. VDR expression has been noted throughout the female reproductive tract (Kwiecinksi *et al* 1989). In vitro studies have shown a direct modulation by vitamin D of estradiol, estrone, and progesterone production in human ovarian cells (Kinuta *et al* 2000; Luk *et al* 2012; Ozkan *et al* 2010). VD as changes in VDR impact on various brain neurotransmitters, and thus suggest a potential role of vitamin D in causing and redressing mood disorders (Kiraly *et al* 2006). We could suppose, even though estrogens and cholecalciferol share similar targets on monoaminergic or another neurotransmitter systems to induce their anxiolytic-like effects, the behavioral manifestations of cholecalciferol are completely different in the adult (3 months) and middle-aged (12–14 months) OVX females. It is likely that cholecalciferol acts through a different mechanisms of action that is sensitive to the age of female rats with long-term absence of ovarian hormones.

In conclusion, the results of this study can be summarized as follows: specific dose of cholecalciferol that was able to induce anxiolytic-like effect is dependent from the age and hormonal state (intact or OVX rats). Further investigations is to be addressed in relation to such issues: whether different effects of cholecalciferol on anxiety-like behavior are dependent from different age of rats, or whether different doses of cholecalciferol on anxiety-like behavior in OVX rats with different age rats might lead to negative versus positive effects. Moreover, further studies are needed to evaluate the association of VD with estrogen-related pathways and

to conduct another chronic experiments together with biochemical studies of these subjects to verify the significance of this study.

## Conclusions

The present data of the preclinical study indicates that chronic cholecalciferol at a dose of 5.0 mg/kg treatment decreased anxiety-related behavior after impairment induced by long-term ovariectomy in the middle-aged female rats with long-term absence of estrogen. The data also indicate that the combination of cholecalciferol at a dose of 5.0 mg/kg and 17β-$E_2$ is more effective than 17β-$E_2$ alone in the middle-aged OVX rats inducing a more synergic anxiolytic-like effects in the EPM and LDT. Furthermore, this is the first study to show a beneficial effect of chronic cholecalciferol at dose of 5.0 mg/kg administration on anxiety-related states induced by long-term ovariectomy in the middle-aged female rats. This work promotes more effective creating of the novel therapeutic targets and strategies for anxiety-like treatment in subjects with long-term estrogen deficiency.

## Acknowledgments

The reported study was funded by Russian S.c.ience Foundation (RSF) accordingly to the research project № 16-15-10053.

***Conflict of Interest.*** *The author declare no conflict of interest.*

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