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Research article

Lavender essential oil ameliorates depression-like behavior and increases neurogenesis and dendritic complexity in rats

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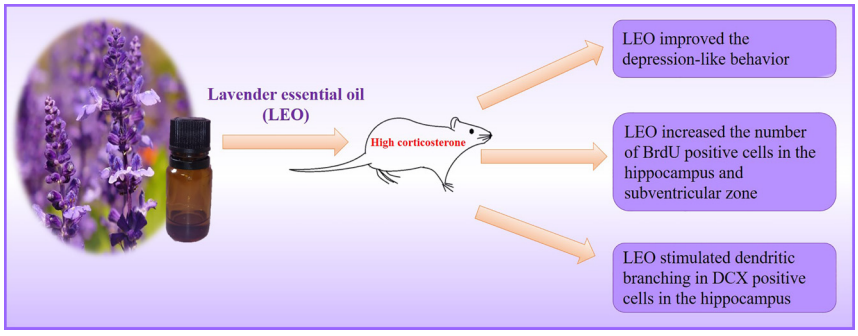
GRAPHICAL ABSTRACT



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ABSTRACT

Depression is a major health issue that causes severe societal economic and health burden. Aromatherapy, a practice that uses essential oils for preventive and therapeutic purposes, represents a promising therapeutic alternative for the alleviation of depressive symptoms. Lavender essential oil (LEO) has been the focus of clinical studies due to its positive effect on mood. An animal model of chronic administration of high dose corticosterone to induce depression- and anxiety-like behavior and reduced neurogenesis was used to explore the biological changes brought by aromatherapy. Twenty-four adult male Sprague Dawley rats were randomly assigned into four groups: Control, corticosterone (Cort) group with high dose of corticosterone, LEO group with daily exposure to LEO by inhalation, and LEO + Cort. At the end of the 14-day treatment period, behavioral tests were carried out. Serum samples were collected 2–3 days after the 14-day period treatment and before perfusion to carry out biochemical analyses to measure BDNF, corticosterone and oxytocin. After perfusion, brains were collected for immunohistochemical analysis to detect BrdU and DCX positive cells in the hippocampus and subventricular zone. Results showed that treatment with LEO ameliorated the depression-like behavior induced by the chronic administration of corticosterone as observed in the LEO + Cort group. Cort treatment reduced the number of BrdU positive cells in the hippocampus and the subventricular zone. Treatment with LEO prevented

Abbreviations: BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; CAM, complementary and alternative medicine; Cort, corticosterone; DCX, doublecortin; DG, dentate gyrus; FST, forced swimming test; LEO, lavender essential oil; NIST, National Institution of Standards and Technology; SIT, social interaction test; SVZ, subventricular zone

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the corticosterone-induced reduction in the number of BrdU positive cells (LEO + Cort group) demonstrating the neurogenic effect of LEO under high corticosterone conditions. Chronic administration of high dose of corticosterone significantly reduced the dendritic complexity of immature neurons. On the contrary, treatment with LEO increased dendritic complexity of immature neurons under high corticosterone conditions (LEO + Cort group). The improved neurogenesis and dendritic complexity observed in the LEO + Cort group demonstrated a clear restorative effect of LEO under high corticosterone conditions. However, 2–3 days after the treatment, the levels of BDNF were upregulated in the LEO and LEO + Cort groups. Furthermore, the concentration of oxytocin in serum, 2–3 days after the treatment, showed to be upregulated in the LEO group alone. The present study has provided evidence of the biological effect of LEO on neuroplasticity and neurogenesis. Also, this study contributes to the understanding of the mechanism of action of LEO in an animal model where depression- and anxiety-like behavior and reduced neurogenesis were induced by high corticosterone administration.

1. Introduction

Depression is a mood disorder characterized by a combination of affective (sadness, anhedonia), cognitive (retardation of thinking, difficulties to concentrate) and physical (changes in appetite, insomnia) symptoms [1–4]. This psychiatric disorder is a major health issue of the century affecting 350 million people around the world [5,6]. While psychosocial interventions are the first line of treatment for less severe patients, antidepressants serve as an important treatment option for patients with more severe signs and symptoms [7]. Even though numerous pharmacological options have been developed since the mid-1950s, there are still concerns about the effectiveness and efficiency of antidepressants [8–10]. The obstacles include non-responders to antidepressant treatment (about 30% of the patients) [3,11]; intolerance to side effects [3,12,13] and delayed onset of therapeutic action which requires a long treatment period [3]. Because of these hurdles, alternative treatment options with potentially higher effectiveness are desired.

Aromatherapy is a popular form of Complementary and Alternative Medicine (CAM) that uses essential oils for the prevention and treatment of a wide variety of health conditions [14,15]. Administration of aromatherapy can be done orally, topically [16,17] or inhaled [18–20]. The evidence from clinical trials shows the promising therapeutic effect of aromatherapy in reducing depressive symptoms [19,21]. Lavender essential oil (LEO), extracted from the flowering heads of *Lavandula aungustifolia* Mill. (syn. *Lavandula officinalis* Chaix), is frequently used in aromatherapy and has been shown to have anxiolytic and antidepressant effects in preclinical and clinical studies [20–23]. However, the mechanism behind the antidepressant effect of LEO remains unexplored. Due to the extensive use of LEO and the clinical evidence available until today, it is worthwhile to explore the effect of LEO on depression and the biological mechanisms that might be implicated.

Neurogenesis, a neuroplasticity process, is an important physiological target for the screening of drugs with potential antidepressant effect [24]. In adult mammals, neurogenesis occurs in the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) along the lateral ventricle [25,26]. Neurogenesis is suggested to be involved in important hippocampal processes including emotional processing [27]. Improved neurogenesis in the hippocampus has been correlated with the use of antidepressants [28]. Therefore, testing whether neurogenesis is a key player in the mechanism of action of LEO is worth to be explored. The brain-derived neurotrophic factor (BDNF) is an important neurotrophic factor that regulates cell survival and has shown a positive effect on neurogenesis [28]. In animal studies, downregulation of BDNF induced by stress has been identified as a causative factor in depression [29,30]. On the other hand, the beneficial effect of antidepressants has been closely linked to the upregulation of BDNF [30]. Another key player to consider in depression is oxytocin, a neuropeptide that modulates social interaction and reproductive behavior [31]. Improvement in social behavior has been reported after nasal administration of oxytocin in animals and human subjects [31,32]. Furthermore, the positive effect on behavior has been observed as a result of oxytocin protective effect against stress [33] and oxytocin-induced stimulation of hippocampal neurogenesis [32,34]. The volatile compounds present in essential oils are absorbed in the olfactory epithelium and the signal travels through the olfactory bulb reaching the limbic system and the hypothalamus which leads to effects on mood [20,22,35]. Because essential oils have shown positive effects on mood [16,36] and oxytocin is an important regulator of social behavior via the olfactory system [31], oxytocin represents an interesting target worthy of investigation. The aim of the present study was to evaluate the effect of LEO on behavior and neurogenesis in an animal model in which depression- and anxiety-like behavior and reduced neurogenesis were induced by chronic administration of high-dose corticosterone. The behaviors evaluated included depression-like and anxiety-like behavior. The mechanism of LEO was explored at a cellular level by focusing on neurogenesis and biochemical analyses.

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley (SD) rats weighing 240–260 g (6–7 weeks of age) were purchased from the Centralized Animal Facility (CAF) at the Hong Kong Polytechnic University. The animals were grouped in 3 rats per cage and kept under 22 ± 2 °C and 12 h dark/light cycle (lights on at 7 a.m.). Food and tap water was provided ad libitum.

2.2. Drugs

LEO extracted from flower tops of *Lavandula aungustifolia* Mill. (syn. *Lavandula officinalis* Chaix) was purchased from DK aromatherapy. A 1% solution of Tween-20 was used to prepare a 2.5% LEO solution that was used in the treatments. Corticosterone was obtained from Sigma Aldrich. For the chemical analysis, LEO was diluted in hexane (Duksan Reagents).

2.3. Chemical characterization of LEO

GC-MS was used to qualitatively characterize the LEO used in the present study. A 1:200 dilution in hexane was prepared. The diluted LEO was analyzed in an Agilent 7890 A GC coupled to a Waters GCT Premier electron ionization-time-of-flight mass spectrometer. Chromatographic separation was performed using an Agilent HP-5 ms GC column (30 m length × 0.25 mm ID × 0.25 µm film thickness) and helium as carrier gas. The temperature program for chromatographic separation was initially held at 30 °C for 1 min, raised to 270 °C with a rate of 10 °C/min, and finally held at 270 °C for 10 min (totally run time 35 min). The electron energy of the electron ionization source of the mass spectrometer was set at 70 eV. The mass spectrometer was first externally calibrated with heptacosa. During data acquisition, internal calibration with the heptacosa was enabled. Identification of LEO was carried out by performing a database search within the National

Institution of Standards and Technology (NIST) library (version 2008) [37,38].

2.4. Experimental design

The effect of LEO on depression- and anxiety-like behavior and neurogenesis was studied in rats [39–41]. Chronic administration of a high dose of corticosterone (40 mg/kg) was used to induce depression- and anxiety-like phenotype and reduced neurogenesis in rats as previously described [34]. The animals were randomly allocated into 4 groups (n = 6 rats per group) and the treatments were administered daily for 14 consecutive days according to the following arrangement: (1) the control group received a subcutaneous (s.c.) injection of propylene glycol (vehicle) and 1 h exposure to a cotton saturated with 1% Tween-20; (2) the corticosterone group (Cort) received a daily s.c. injection of 40 mg/kg corticosterone and 1-hour exposure to a cotton saturated with 1% Tween-20; (3) the LEO group was exposed to a cotton saturated with 2.5% LEO and received a s.c. injection of propylene glycol; (4) the LEO + Cort was administered a s.c. injection of 40 mg/kg corticosterone and exposed to a cotton impregnated with 2.5% LEO. Corticosterone was administered 20 min after exposure to either vehicle or LEO [42]. The administration of the different treatments was done daily for 14 consecutive days between 11:00 and 14:00. An intraperitoneal injection of 50 mg/kg/day BrdU was administered to all the animals at days 12, 13 and 14 in order to label the proliferating cells (Taupin, 2007). The behavioral tests were performed on days 15 and 16. Two to 3 days after the 14-day period of treatment was stopped, plasma samples were collected. On day 16 and 17, intracardial perfusion was carried out between 10:00–17:00 and the brains and adrenal glands were collected.

2.5. Inhalation equipment and treatment administration

Individually, rats were introduced into the inhalation equipment made of an acrylic fiber box (42 × 30 × 29 cm). The front and back walls of the chamber had four holes (2 cm diameter/hole) with a perforated acrylic fiber wall placed 3 cm apart from the front and back walls. The cotton soaked with the respective treatment was placed between the outer and inner walls and out of the animals' reach (Fig. 1). In order to allow proper ventilation in the chamber, the inner and top wall contained 25 small holes [43]. All animals were exposed to 1 ml of the vehicle or 1 ml of 2.5% LEO for 1-hour. The exposure time was established based on previous studies (Linck et al., 2010; Hritcu et al., 2012; Hancianu et al., 2013). Two different chambers were used: one exclusively assigned for the treatment with vehicle and the other one for the treatment with LEO. After each animal was exposed to the treatment, the equipment was cleaned with 70% ethanol.

2.6. Behavioral tests

2.6.1. Forced swimming test (FST)

The total number of animal used was 24 (n = 6 rats per group). The assessment of depressive-like behavior was carried out using the FST as previously described [39,40,44,45]. The time for the assessment of depression-like behavior by the FST was between 11:00-14:00. The equipment for the FST consisted of a vertical transparent cylinders (40 cm height × 30 cm diameter) filled with tap water (room temperature) at a depth of 30 cm. The level of the water used was enough to avoid that the animals reached the bottom of the apparatus with neither the hind paws nor the tail. Two sessions were involved in the FST: (1) a 15 min long pre-test to induce a state of helplessness followed by (2) a 10 min test carried out 24 h after the pre-test. The second session of the test was video recorded to be scored by an observer blinded to the treatment groups. The time spent performing the following behaviors was scored: (1) Immobility or floating with absent signs of struggling and minimal to no movement to keep from drowning; (2) swimming behavior characterized by active movement of the forelimbs or hind limbs in a paddling manner, the movement is more than necessary to keep the head above the water, but less motion as the one shown in climbing/struggling behavior; (3) climbing or struggling characterized by fast and vigorous movements of the forelimbs and hind limbs breaking the surface of the water to try to get out of the container. High immobility time is an indication of depression-like behavior [39,46].

2.6.2. Social interaction test (SIT)

The total number of animal used was 24 (n = 6 rats per group). Anxiety-like behavior was assessed as previously described using the SIT [39,47]. The time for the assessment of anxiety-like behavior by the SIT was between 11:00–14:00. The open-field consisted of an arena of 72 cm length × 72 cm width × 40 cm depth with a camera mounted on top of the field to record the behavior to be scored by an experimenter blinded to the treatment groups. Two unfamiliar rats were placed in the test arena for 10 min and the initiation of the following behaviors was scored (1) non-aggressive behavior (positive social behavior) including sniffing, following, crawling, social play, grooming; (2) aggressive or defensive behavior which comprises kicking, boxing, wrestling, biting; (3) no interaction identified when the rats were apart from each other including sniffing or exploring the box. Increased anxiety-like behavior is shown with a low number of positive social behaviors [39,47,48].

2.7. Animal perfusion and tissue processing

Before animal perfusion (2–3 days after the 14-day treatment period was stopped), samples from truncal blood were taken and centrifuged at 1000 × *g* for 15 min to collect the serum (the total number of animals was 24, n = 6 animals per group). Serum samples were stored at −80 °C. The serum was used to carry out biochemical analyses to determine the concentration of BDNF, corticosterone, and oxytocin. A lethal dose of sodium pentobarbital (200 mg/kg, intraperitoneal) was administered to the animals right after the collection of the serum samples (the total number of animal used was 24, n = 6 rats per group). Transcardial perfusion using 4% paraformaldehyde was carried out as previously described [49,50]. Brain samples were post-fixed overnight at 2–8 °C in 4% paraformaldehyde. After post-fixation, the brain tissues were immersed in cryoprotectant solution (30% sucrose solution in 0.1 M phosphate-buffered saline) and stored at 2–8 °C [51]. The adrenal glands were also collected and stored at −80 °C. Coronal brain sections, 40 µm thick, were prepared in a 1-in-12 series of consecutive sections using a Cryotome E (Thermo Electron Corporation) and stored in the cryoprotectant solution at −20 °C. Sections of the hippocampus and SVZ were fixed on glass slides coated with gelatin for immunostaining.

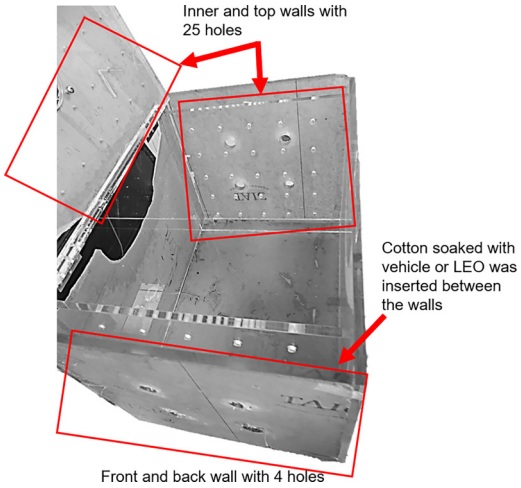

Fig. 1. Inhalation equipment.

2.8. Immunoperoxidase staining of BrdU positive cells

Detection of BrdU (neuronal cell proliferation marker) positive cells was performed as previously described (Lau et al., 2012). The total number of animal used was 24 (n = 6 rats per group). Hippocampal and SVZ sections mounted on glass slides were rinsed (3 times for 10 min) with 0.01 M PBS. The slides were incubated in preheated citrate buffer at 90 °C for antigen retrieval. DNA denaturation was carried out by incubating the slides in 2 N HCl at 40 °C for 30 min. Neutralization of the acid was done by incubating the slides in 0.1 M borate buffer for 15 min at room temperature. After neutralization, the slides were washed (3 times for 10 min) with 0.01 M PBS before incubation with 1:1000 mouse anti-BrdU antibody (Roche) overnight. After incubation, slides were washed (3 times for 10 min) with 0.01 M PBS and the slides were incubated with 1:200 biotinylated goat anti-mouse antibody (Dako) for 2 h at room temperature. After incubation with the secondary antibody, the slides were washed (3 times for 10 min) with 0.01 M PBS. Signal amplification was performed using an avidin-biotin complex system (Vector) to visualize the BrdU labeled cells with diaminobenzidine hydrochloride as the chromogen [52,53]. Immunostained sections were air-dried, counter-stained with 10% eosin in 70% ethanol for 3 min, dehydrated as follows (3 min incubation in every solvent): 2 times in 90% ethanol, 3 times in 100% ethanol and 3 times in xylene at room temperature. DPX mounting media was used to coverslip the slides (Thermo Scientific) [54].

2.9. Immunoperoxidase staining of DCX positive cells

Detection of immature neurons was carried out by immunostaining of doublecortin (DCX, a protein expressed by immature neurons) positive cells as previously described [52]. The total number of animal used was 24 (n = 6 rats per group). The DCX immunostaining protocol is similar to the BrdU protocol except that the incubations with HCl and borate buffer were skipped and the primary and secondary antibody used were rabbit anti-DCX antibody (1:300, Cell signaling) and goat anti-rabbit biotinylated antibody (1:200, Dako) respectively [52].

2.10. Quantification of BrdU and DCX positive cells

Quantification of BrdU and DCX positive cells was done using the optical fractionator probe in a Stereo Investigator system (version 11, MBF Bioscience) as previously described [34]. Brain sections from six animals per group were analyzed including 6 hippocampus sections and 4 SVZ sections per animal. The BrdU positive cells were identified as dark-brown round spots along the dentate gyrus of the hippocampus and the SVZ. In the hippocampus, the total number of BrdU cells, automatically calculated by the software, was estimated by counting the BrdU positive cells in 6 sections, and then calculating the average number of cells, and multiplying the average number of cells by 12 since the systematic sampling was done on every 12th section. In the SVZ, the average number of BrdU positive cells per section was calculated by taking the total number of cells counted per section (4 SVZ sections in total) and calculating the average number of cells. The DCX

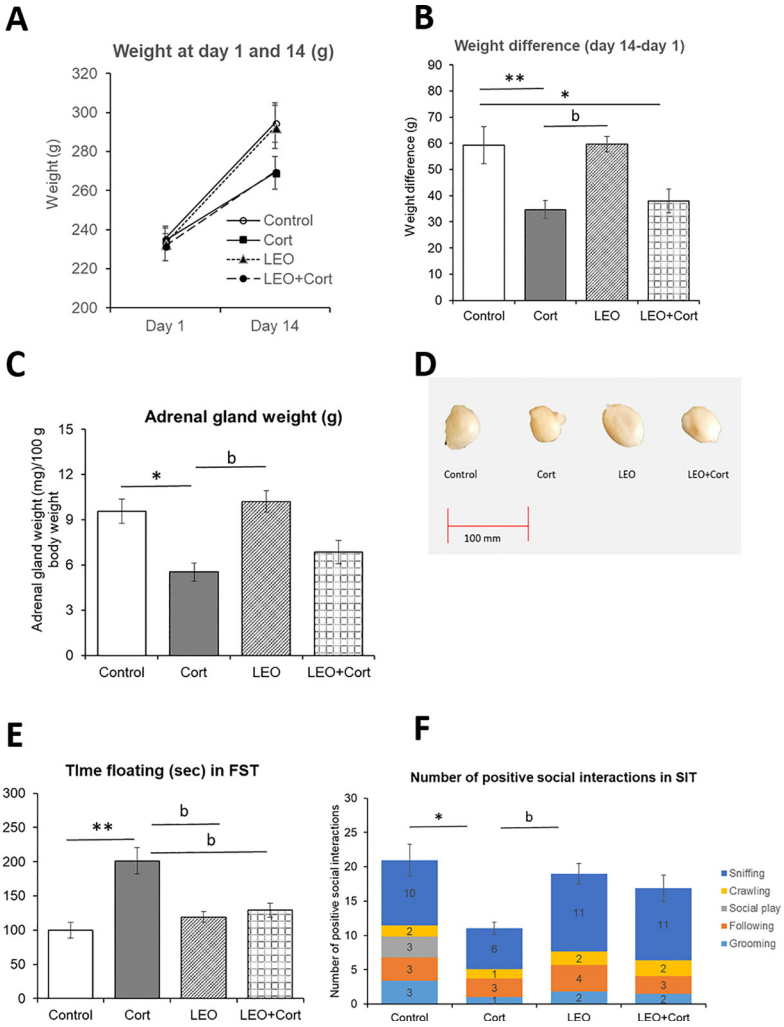

Fig. 2. *LEO reverted the high corticosterone induced depression-like and anxiety-like behavior but did not revert the decreased weight gain induced by corticosterone treatment.* (**A**) Body weight on day 1 (Kruskal-Wallis test) and 14 (One-way ANOVA). No significant difference observed among the groups. (**B**) The weight difference between day 1 and day 14 (One-way ANOVA). Treatment with LEO did not have any effect on weight gain. Treatment with CORT decreased weight gain when compared to the control and LEO group, and the effect was not reverted by co-treatment with LEO as observed in the LEO + CORT group. (**C**) Treatment with LEO restored the low weight of the adrenal gland induced by CORT treatment to an average weight showing no signifficant difference between the LEO + CORT and control group (One-way ANOVA with Tukey posthoc test). However, there was also no significant difference between the CORT and LEO + CORT group. Treatment with LEO alone did not affect the adrenal gland weight. (**D**) Representative images of the adrenal gland per treatment group. (**E**) LEO decreased the depression-like behavior induced by corticosterone treatment shown by reduced immobility time in the FST as showed when comparing the CORT and LEO + CORT groups (Kruskal-Wallis with Mann-Whitney U test). Furthermore, LEO treatment alone did not affect the immobility time. (**F**) LEO reduced the corticosterone-induced anxiety-like behavior observed in a higher number of positive social interactions and treatment with LEO alone showed no significant difference when compared to the control group (One-way ANOVA with Tukey posthoc test). Results expressed as Mean ± SEM; *p < 0.05 and **p < 0.01 when compared to the control group; ^{b} p < 0.05 when compared to the CORT group.

positive cells were identified as the whole cell, including the cell body and dendrites, and they were stained. The counting procedure used to quantify the BrdU positive cells was used to quantify the DCX positive cells in both regions of the brain. The following settings were used in the optical fractionator probe for counting both BrdU and DCX positive cells: dissector height of 25 µm and 60 X 60 µm sampling site size. In the hippocampus, the total number of positive BrdU and DCX cells was expressed as mean ± SEM. In the SVZ, the average number of BrdU or DCX positive cells per section was expressed as mean ± SEM.

2.11. Dendritic complexity of immature neurons

Sholl analysis, a tool used to determine changes in the neuronal dendritic arborization, involves a systematic procedure to count the number of intersections of the dendrites to concentric circles drawn at fixed distances from the soma [55,56]. The assessment of spatial distribution and dendritic complexity was carried out by tracing cells exhibiting tertiary of higher arborization with untruncated dendrites and perpendicular orientation [52,57]. Images of the selected neurons (40x magnification) were captured to be processed in NeurphologyJ plugin for ImageJ software [57,58]. A total number of 10 neurons per animal were selected (60 neurons per treatment group, the total number of animal used was 24, n = 6 rats per group; therefore, 10 neurons per animal were selected) [59]. The fixed settings for the Sholl analysis include a starting point of 10 µm, a step size of 10 µm and an ending radius of 200 µm. Additional measurements comprise the distance from the soma to each intersection and the total number of intersection that corresponds to the number of times the dendrites intersected the fixed concentric circles. The Sholl analysis was performed by an experimenter blinded to the treatments. A high dendritic complexity is reflected in a high number of intersections.

2.12. Biochemical analyses in serum

The serum samples were collected 2–3 days after the 14-day treatment period and before animal perfusion to carry out biochemical analyses. The serum concentration of BDNF (Millipore), corticosterone (Enzo Life Science) and oxytocin (Enzo Life Science) was measured by ELISA kits adhering to the manufacturer instructions. The total number of animal used was 24 (n = 6 rats per group).

2.13. Statistical analysis

The difference among treatment groups was analyzed using the SPSS software (version 13.0). One-way ANOVA with Tukey posthoc test was used when the data to be analyzed meet the criteria of homogeneity and equal variance. In the event that either the assumptions of normality, checked by Shapiro-Wilks test, or homogeneity of variance, checked by Levene's test, were not met, the non-parametric tests Kruskal-Wallis and Mann-Whitney U were used. A p-value < 0.05 was

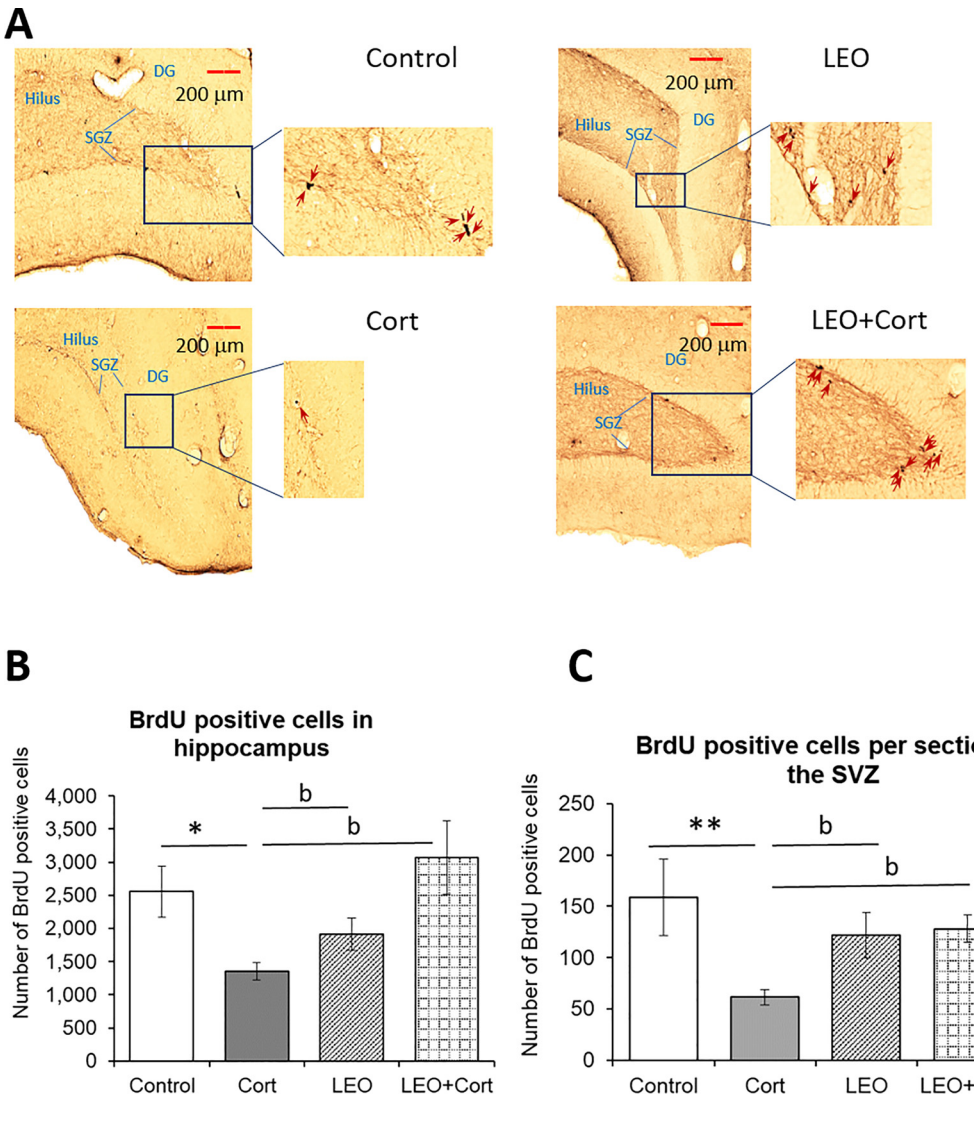

Fig. 3. *LEO reverted the CORT-induced reduced number of BrdU positive cells in the hippocampus and SVZ.* (**A**) Representative images of the effect of the treatments on the total number of BrdU positive cells in the hippocampus; (**B**) treatment with LEO reverted the corticosterone-induced decreased total number of BrdU positive cells in the hippocampus; (**C**) in the SVZ, LEO reverted the decreased number of BrdU positive cells per section induced by Cort treatment. Results are expressed as Mean ± SEM; *p < 0.05 and **p < 0.01 when compared to the control group; [b] p < 0.05 when compared to the Cort group; Kruskal-Wallis with Mann-Whitney U test. DG, Dentate gyrus; SGZ, subgranular zone. The arrow indicates positive staining.

used to indicate statistical significance.

3. Results

3.1. Chemical characterization of LEO

The chemical profile of the LEO used in the present study was determined by qualitative GS-MS analysis. Linalool, β-pinene, o-cymene, α-ocimene, β-ocimene, allo-ocimene, α-terpineol, camphene, δ-3-Carene, γ-terpinene, terpinen-4-ol, Caryophyllene, β-humulene were identified in LEO. The chemical characterization of LEO is in line with the chemical profile of LEO reported in previous studies [37,38,60–62].

3.2. Body weight and adrenal gland weight

Before the treatment started, the body weight of the animals was measured and no significant difference in the body weight was found among the groups (Fig. 2 A). At the end of the treatment, no difference in the weight of the animals was observed. However, a significant difference in the weight gain was found (Fig. 2 B; $p = 0.001$). A significant difference in the weight gain was observed between the control (mean ± SEM; 59.3 g ± 7.11) and Cort (34.6 g ± 3.4) groups in which the Cort group showed lower weight gain. Similarly, a significant difference ($p = 0.024$) was found between the LEO + Cort (38 g ± 4.57) and the control group (59.3 g ± 7.11). On the contrary,

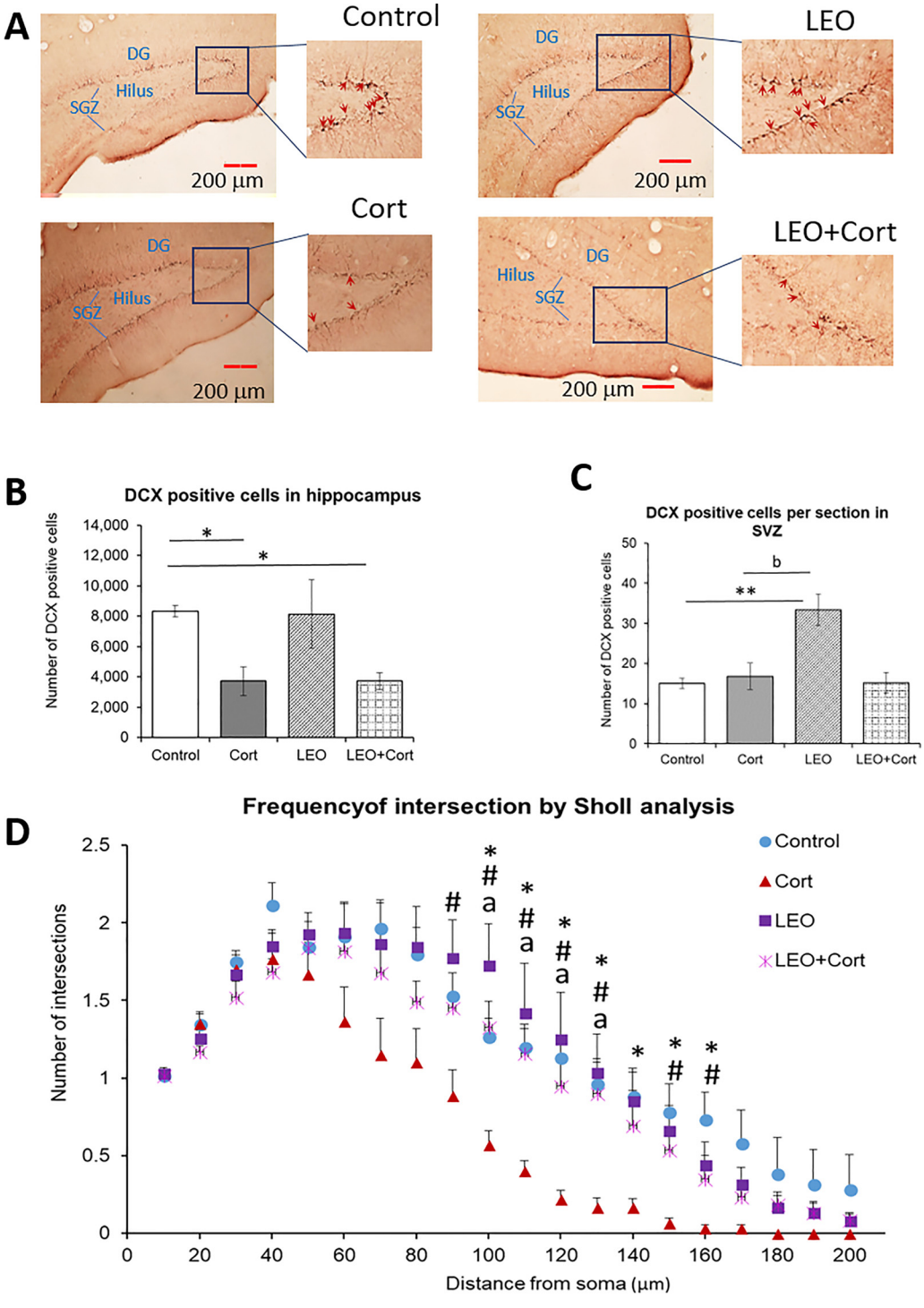

Fig. 4. LEO treatment did not revert the corticosterone-induced reduced number of DCX positive cells in the hippocampus, but LEO alone increased the number of DCX positive cells per section in the SVZ. Furthermore, treatment with LEO restored the lower-dendritic complexity-induced by Cort treatment. (**A**) Representative images of the effect of the treatments on the total number of DCX positive cells in the hippocampus; (**B**) LEO alone did not affect the total number of positive DCX cells and LEO did not revert the corticosterone-induced reduction in the number of DCX positive cells in the hippocampus; (**C**) in the SVZ, treatment with LEO alone significantly increased the number of DCX positive cells per section. Cort and LEO + Cort treatments did not show any effect on the number of DCX positive cells in the SVZ. (**D**) Treatment with LEO increased the dendritic complexity in the LEO + Cort group when compared to the Cort group. Therefore, LEO reverted the lower-dendritic-complexity induced by Cort treatment. No statistically significant difference was observed in the number of intersections among the control, LEO and LEO + Cort groups. Results are expressed as Mean ± SEM; *$p < 0.05$ and **$p < 0.01$ when compared to the control group; ^{b} $p < 0.05$ when compared to the Cort group; One-way ANOVA with Tukey posthoc test and non-parametric tests Kruskal-Wallis and Mann-Whitney U (when applicable). In the Sholl analysis, $p < 0.05$ and statistically significant difference among the groups are shown as follows: *: Control vs Cort; #: Cort vs LEO; a: Cort vs LEO + Cort. DG, Dentate gyrus; SGZ, subgranular zone. The arrow indicates positive staining.

treatment with LEO alone (59.66 g ± 2.92) did not show any difference when compared to the control group (59.3 g ± 7.11). A significant difference was observed among the groups in the adrenal gland weight (Fig. 2 C and D; p = 0.001). The lowest adrenal gland weight was found in the Cort group (5.53 g ± 0.61) and was statistically different when compared with the control group (p = 0.04). No significant difference in the adrenal gland weight was found when comparing the control (9.56 g ± 0.79) and LEO group (10.22 g ± 0.71). Interestingly, the comparison between the control (9.56 g ± 0.79) and LEO + Cort (6.86 g ± 0.78) showed no significant difference (p = 0.076).

3.3. Behavioral tests

The time spent floating in the FST showed a significant difference among the groups in the FST (Fig. 2 E; p = 0.004). The longest time spent floating was observed in the Cort group (201.33 s ± 19.19) indicating depression-like behavior and it was statistically different (p = 0.004) when compared with the control group (99.60 s ± 11.52). The findings showed more severe depression-like behavior in the Cort group. Co-treatment with LEO (LEO + Cort group) reverted the depression-like behavior induced by administration of corticosterone and this was shown as decreased time spent floating (129 s ± 10.05) which was comparable to the floating time in the control group (99.60 s ± 11.52). Also, the time spent floating in the LEO group (119.25 s ± 8.04) was comparable to that of the control group (99.60 s ± 11.52). In the SIT, no aggressive behavior was observed in any of the animals (total number of animals = 24, 6 animals per group). The SIT assesses anxiety-like behavior and a significant difference among the groups was observed with regard to the number of positive social interactions (Fig. 2 F; p = 0.013). The total number of positive social interactions was significantly reduced in the Cort group (9.67 ± 2.30) when compared with the control group (p = 0.035, 17 ± 2.30) indicating increased anxiety-like behavior in the Cort group. Treatment with LEO alone (18 ± 1.50) did not affect the number of positive social interactions and showed statistically significant difference when compared to the Cort group (p = 0.013). Co-treatment with LEO showed an increase in the number of positive social interactions when comparing the LEO + Cort group (16.40 ± 2.15) with the Cort group (9.67 ± 2.30) but the difference was not significant (p = 0.074). However, the increased number of positive interactions in the LEO + Cort group was comparable to the control group showing no difference.

3.4. BrdU and DCX positive cells

A significant difference in the total number of BrdU positive cells in the hippocampus was observed (Fig. 3A and B; p = 0.021). Cort treatment significantly reduced the cell proliferation in the hippocampus as reflected in the reduced number of BrdU positive cells (1354.8 ± 130.38) when compared with the control group (2556 ± 389.46) (p = 0.016). Treatment with LEO alone did not affect the number of BrdU positive cells (1917.75 ± 252.14). Interestingly, LEO treatment reverted the Cort-induced reduction in the number of BrdU positive cells in the LEO + Cort group (3069.33 ± 557.85; p = 0.016). The findings demonstrate that treatment with LEO protected the hippocampus against the negative effects induced by Cort by stimulating cell proliferation. In the SVZ, the number of BrdU positive cells showed a significant difference (Fig. 3C; p = 0.006). The number of BrdU positive cells was significantly reduced in the Cort group (61.5 ± 7.62) when compared with the control group (158.80 ± 37.66, p = 0.004). Treatment with LEO alone did not affect the number of BrdU positive cells in the SVZ (122 ± 22.26). However, LEO showed a positive effect on cell proliferation in the SVZ by reverting the Cort-induced reduction in the number of BrdU positive cells as shown in the LEO + Cort group (128.20 ± 13.73) when compared with the control group (158.80 ± 37.66).

DCX is a marker of immature neurons. In the hippocampus, a significant difference was found in the total number of DCX positive cells (Fig. 4A and B; p = 0.011). Treatment with Cort had a negative impact on the number of immature neurons in the hippocampus (3720 ± 948.68; p = 0.042) and it showed a significant difference when compared with the control group (8335.33 ± 372.45). Treatment with LEO alone did not affect the number of DCX positive cells (8131.75 ± 2247.03) when compared with the control group. However, a significant difference was observed in the number of DCX positive cells in the LEO + Cort group (3716 ± 552.21) when compared with the control group (8335.33 ± 372.45; p = 0.041), demonstrating that treatment with LEO did not revert the Cort-induced negative effect on the number of immature neurons. In the SVZ, a statistically significant difference in the number of DCX positive cells was observed (Fig. 4C; p = 0.001). Treatment with LEO alone (33.40 ± 3.95) caused a significant increase in the number of immature neurons in comparison with the control group (15 ± 1.29; p = 0.001). The Cort group (16.83 ± 3.28) and LEO + Cort group (15.17 ± 2.54) did not show any statistically significant difference when compared with the control group (15 ± 1.29). However, a significant difference between the Cort and LEO group was observed.

3.5. Dendritic complexity of immature neurons

The dendritic complexity of DCX positive cells showed interesting results in the Sholl analysis (Fig. 4D). A significant difference in the dendritic complexity was observed between the control and Cort group in which the Cort group showed a lower number of intersections. No significant difference between the control, LEO and LEO + Cort groups was found indicating a positive effect of LEO to revert the decreased dendritic complexity induced by Cort.

3.6. BDNF, corticosterone and oxytocin levels in serum

Serum samples were collected 2–3 days after the 14-day teratment period to measure BDNF, corticosterone and oxytocin levels. Caution should be taken when interpreting the biochemical analyses carried out in serum as the 2–3 days post-treatment delay to collect the serum sample could not allow direct causal correlation between the treatment and the concentration of BDNF, corticosterone and oxytocin. However, the biochemical analysis carried out provide some information that can be valuable for further studies on the mechanism of action of LEO in depression. The BDNF levels in serum were significantly different among the treatment groups (Fig. 5 A; p = 0.005). High BDNF levels were observed in the Cort (5464.36 pg/ml ± 843.98, p = 0.016), LEO (6871.46 pg/ml ± 974.01, p = 0.016) and LEO + Cort groups (3414.95 pg/ml ± 453, p = 0.029) when compared to the control group (442.03 pg/ml ± 22.38). The level of BDNF in the LEO group was increased after 2–3 days that the treatment was stopped (6871.46 pg/ml ± 974.01) when compared with the LEO + Cort group (3414.95 pg/ml ± 453, p = 0.016). However, no significant difference was found between the Cort (5464.36 pg/ml ± 843.98) and LEO groups (6871.46 pg/ml ± 974.01). A significant difference was observed in the corticosterone levels in serum (Fig. 5 B; p = 0.009). Two to 3 days after the 14-day treatment period was stopped, it was observed that the Cort group had a lower level of corticosterone (142.57 ng/ml ± 27.99) when compared with the control group (340.99 ng/ml ± 51.89, p = 0.004). The corticosterone levels in serum were similar in the control (340.99 ng/ml ± 51.89), LEO (294.68 ng/ml ± 19.86) and LEO + Cort groups (215.55 ng/ml ± 39.31). However, a significant difference was observed between LEO (294.68 ng/ml ± 19.86) and Cort group (142.57 ng/ml ± 27.99, p = 0.004). The level of oxytocin was also measured 2–3 days after the treatment period was stopped. A statistically significant difference among the treatment groups was observed (Fig. 5 C; p = 0.022). The concentration of oxytocin in the Cort group

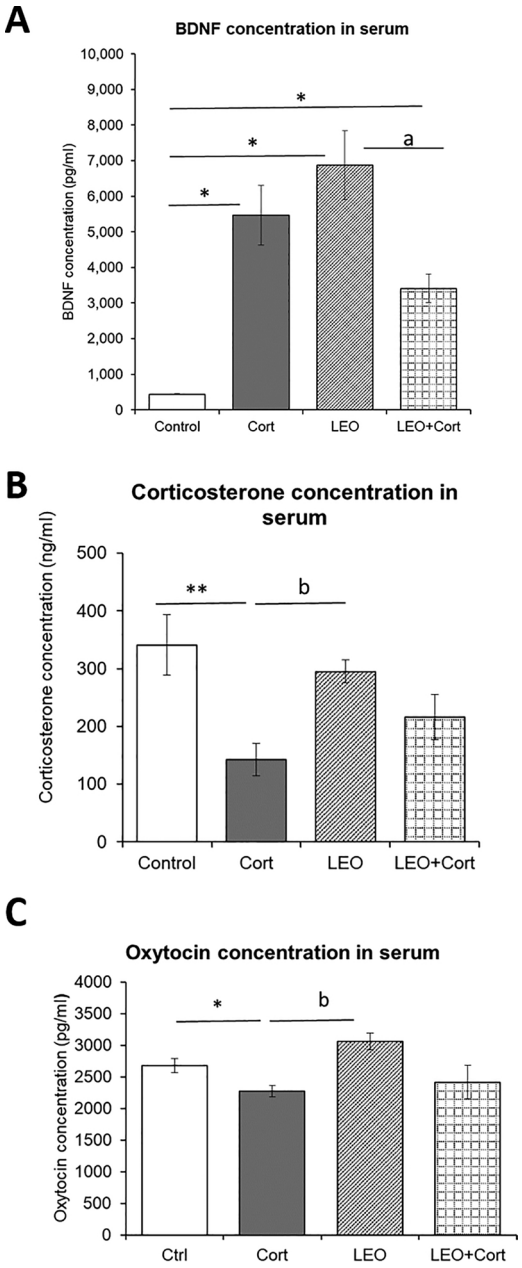

Fig. 5. Two to three days after the treatment was stopped, an increase in the levels of BDNF and oxytocin were observed in the LEO group when compared to the control group. (**A**) BDNF concentration in serum. After the 14-day treatment was stopped (2–3 days after) the concentration of BDNF in serum was high in the Cort, LEO and LEO + Cort groups. (**B**) Corticosterone concentration in serum. (**C**) Oxytocin concentration in serum. Two to three days after the treatment was stopped, the levels of oxytocin in serum were higher in the LEO alone group when compared to the control group. Results are expressed as Mean ± SEM; *$p < 0.05$ and **$p < 0.01$ when compared to the control group; [a] $p < 0.05$ when compared to the LEO group; [b] $p < 0.05$ when compared to the Cort group; Kruskal-Wallis and Mann-Whitney U.

(2273.04 pg/ml ± 93.44) was lower than the concentration in the control group (2677.35 pg/ml ± 110.55; $p = 0.038$). In the LEO group, 2–3 days after the treatment was stopped, the level of oxytocin observed was higher (3057.69 pg/ml ± 132.23) when compared with the Cort group (2273.04 pg/ml ± 93.44, $p = 0.004$). No significant difference was found when comparing the control, LEO and LEO + Cort groups.

4. Discussion

The present study demonstrated a positive effect of LEO in alleviating depression-like behavior, increased neurogenesis and dendritic complexity in an animal model in which depression- and anxiety-like behavior and decreased neurogenesis were induced by chronic administration of corticosterone. Treatment with LEO increased the number of BrdU-positive cells in the hippocampus and SVZ and reverted the corticosterone-induced suppression of neurogenesis. In the hippocampus, LEO improved the dendritic complexity in the LEO + Cort group, but the number of immature neurons was not increased when compared with the Cort group. In the SVZ, treatment with LEO alone increased the number of DCX positive cells. A higher concentration of BDNF and oxytocin in the LEO alone group was observed 2–3 days after the 14-day treatment period was stopped. Both BDNF and oxytocin are relevant in the regulation of the neurogenesis process. Although the concentration of BDNF and oxytocin detected 2–3 days after the treatment period was stopped did not show any significant difference between the LEO + Cort and Cort group, the increase observed in the LEO group may indicate a promising effect of LEO that should be investigated more in detail. For instance, a longer treatment period, 3–4 weeks instead of 2 weeks, could be implemented and serum samples could be immediately taken after the treatment period instead of 2–3 days after stopping the administration of the treatments. However, the findings suggest a novel perspective with respect to the treatment effect of LEO highlighting that neurogenesis may be an underlying mechanism. The findings enhance understanding of the pharmacological effects of LEO and support its potential clinical application as an antidepressant. However, further investigation is needed.

4.1. Chemical characterization of LEO, and its effect on body weight and adrenal gland weight

The chemical characterization of LEO used in the present study is in accordance with previous reports [38,60,61]. LEO contains a complex mixture of volatile compounds. Linalool and linalyl acetate are the most commonly found components in LEO [23,38]. Of these, linalool has been proposed as the most biologically active compound in LEO [14].

Cort treatment significantly reduced the body weight gain in the Cort and LEO + Cort groups. The results agree with previous studies that attributed the weight loss to the chronic administration of a high dose of corticosterone in rats. Treatment with LEO did not affect the body weight gain. However, a study conducted by Shen et al. (2005) showed the opposite findings [63]. The contrast in findings may be attributed to the shorter duration of Shuen et al.'s study when compared with the present study. The adrenal gland weight was significantly lower in the Cort group which is consistent with the findings of previous studies where decreased adrenal gland weight was observed after corticosterone treatment [64]. The reduced adrenal gland weight is associated with hypoactivity of the hypothalamic-pituitary-adrenal axis (HPA axis). Corticosterone is the end product of the activation of the HPA axis which mimics the physiological stress response [65]. The adrenal gland is an important component of the HPA axis, a physiological regulator system of the stress response that uses glucocorticoids such as corticosterone, as an effector molecule [66]. Upon activation, the HPA axis triggers the release of corticotropin-releasing factor (CRF) which in turn stimulates the synthesis and release of glucocorticoids from the adrenal gland. In rodents, high concentrations of circulating corticosterone cause a negative feedback in the HPA axis that inhibits the release of CRF [67]. At high corticosterone conditions, the activation of the HPA axis is downregulated [67] and lower adrenal gland weight is observed as a result of adrenal gland atrophy caused by chronic administration of corticosterone [68,69]. Our study demonstrated that co-treatment with LEO showed an increase in adrenal weight, which reverts the effect of the Cort-induced low adrenal gland weight.

4.2. Effect of LEO on depression-like and anxiety-like behavior

Co-treatment with LEO attenuates the depression-like behavior only under stress conditions (LEO + Cort group). Cort treatment significantly increased the time that animals spent floating in the FST, which is an indication of depression-like behavior [39,70]. Exposure with LEO and linalool reduced the depression-like behavior in previous animal studies [71–73]. The findings of the present study showed the reduction of the depression-like behavior caused by LEO exposure, thereby reverting the negative behavioral effects induced by Cort treatment in the LEO + Cort group.

In the SIT, treatment with Cort decreased the number of positive social interactions as previously reported [34]. In clinical studies, high levels of stress or chronic exposure to stress hinders individual engagement in social interactions due to social anxiety [74]. An increased number of positive social interactions was found after treatment with LEO in the LEO + Cort-treated group to levels comparable to those of the control group. The results in the SIT found in the present study suggest that treatment with LEO under stress conditions (LEO + Cort group) improves positive social interactions. The increased number of positive social interactions in the LEO + Cort group was not enough to show a significant difference when compared with the Cort group. However, the trend to increase the number of positive social interactions in the LEO + Cort group is clearly suggesting that further investigation should be carried out using a longer treatment period.

In previous studies, an increased engagement in social interaction was observed when animals were treated with linalool by inhalation [75]. Again, an increased social interaction was observed by Kumar (2013) in a dose-dependent manner when administering the LEO formulation Silexan. Therefore, improved social interaction has been previously reported and this is corroborated by the results observed in the present study. We demonstrated that treatment with LEO increased the number of positive social interactions in the LEO + Cort group to a level comparable to the one of the control group. The results suggest a potential protective action of LEO against the anxiogenic effect of Cort, but further investigation is needed using a longer duration of the treatment in order to confirm the effect of LEO on anxiety-like behavior.

4.3. Effect of LEO on the number of BrdU and DCX positive cells

Hippocampal neurogenesis is subjected to regulation by glucocorticoids [76] as a high density of mineralocorticoid and glucocorticoid receptors are present in the DG of the hippocampus [77]. Although it has been hypothesized that aromatherapy may promote neurogenesis in the hippocampus [35,78], no evidence concerning the effect of LEO on neurogenesis has been identified. To the best of our knowledge, the present study is the first to report the effect of LEO on neurogenesis. Suppression of newborn cells, labeled by BrdU, was observed in the Cort group which is accordance with the stress-mediated decreased cell proliferation phenomenon described above [64,79]. Remarkably, treatment with LEO reverted the suppression of cell proliferation induced by Cort treatment (LEO + Cort group) indicating that LEO protected hippocampal neurogenesis from the effect of Cort. Antidepressants increase cell proliferation in the hippocampus restoring the suppressed neurogenesis observed in stress and depression [80]. The restoration of the number of BrdU positive cells in the hippocampus observed in the LEO + Cort treatment showed that LEO exhibited an effect on the hippocampus similar to the effect caused by antidepressants. The findings of the present study suggest that LEO may be a promising treatment alternative for depression.

Cort treatment suppressed cell proliferation in the SVZ. Similar results have been reported in previous studies as well [81,82]. We observed that LEO treatment protected the SVZ against the suppressed cell proliferation induced by Cort treatment as it was observed in the LEO + Cort group. LEO treatment alone did not increase the number of BrdU positive cells which suggests that LEO treatment only increased cell proliferation under stress conditions (LEO + Cort group). The SVZ is another neurogenic region where new neurons are continually added to the olfactory bulb within the mammalian brain [83]. The SVZ is a distinctive neurogenic region apart from the hippocampus. For this reason, the SVZ has been suggested to play a significant role in neural plasticity within the adult brain [83]. The results from the present study demonstrated that LEO stimulates cell proliferation not only in the hippocampus but also in the SVZ under high corticosterone conditions (LEO + Cort group). This seems to indicate an interesting effect of LEO on SVZ neurogenesis.

Treatment with LEO did not improve the suppression of the number of DCX cells induced by Cort treatment in the hippocampus as observed in the LEO + Cort group. However, LEO significantly increased the number of DCX positive cells in the SVZ when administered alone, but no change was observed under high corticosterone conditions (LEO + Cort group). The effect of LEO on the number of immature neurons in the SVZ suggests that LEO stimulates cell differentiation, but the effect is not present under stress conditions. The dendritic complexity of DCX positive cells determined by the Sholl analysis showed a decrease in the frequency of intersections in the Cort group. This is consistent with previously reported findings [34,84–86]. In the early post-mitotic maturation phase in hippocampal neurogenesis, immature neurons increase the dendrite and axon extensions, thereby facilitating an increase in synaptic plasticity [87]. Antidepressants have been shown to reverse the harmful effects of stress on synaptic and dendritic structures [88]. Therefore, the improved dendritic complexity within the hippocampus in the LEO + Cort group suggests that LEO treatment stimulates dendritic branching. In the present study, LEO reverted the negative effect of corticosterone on dendritic complexity as shown in the LEO + Cort group.

4.4. Effect of LEO on systemic BDNF, corticosterone and oxytocin levels

As mentioned above, the interpretation of the biochemical analysis of BDNF, corticosterone and oxytocin should be cautious since the serum samples were taken 2–3 days after the 14-day treatment period was stopped. However, the data provided could shed some light for the design on future studies on the mechanism of action of LEO as antidepressant. BDNF is a growth factor present in most tissues, highly expressed in the hippocampus and cortical region of the brain and found in blood [89,90]. It is an important regulator of survival, differentiation, and growth functions in the brain [91]. BDNF can cross the blood-brain barrier and it is found in serum, plasma and stored in high amounts in platelets [29,92]. Changes in BDNF expression in the brain have been correlated with BDNF levels in serum [29,93]. Clinical studies focused on the measurement of circulating BDNF have reported lower levels of BDNF in patients with major depression [89]. Due to its presence in serum and its correlation with the disease states, BDNF has been considered a promising biomarker for brain disorders [89]. Furthermore, not only has the treatment with antidepressants increased the levels of BDNF in serum, but also other neuro-rehabilitation approaches have shown the same effect [89]. The peripheral administration of BDNF has shown antidepressant effect similar to the increased BDNF in serum observed after antidepressant treatment [93]. Several studies have demonstrated that the upregulation of BDNF increases neurogenesis [28,90,94] since BDNF increases the survival of newborn cells in the dentate gyrus of the hippocampus [90]. Although several molecular pathways regulated by BDNF have been studied, it is not known which one is the most crucial for the antidepressant effects observed in animal models [95]. Yet, it is still important to explore the involvement of BDNF in the mechanism of action of novel treatment options for depression. Not only is the direct infusion into the hippocampus vital but also the peripheral administration of BDNF has been shown to regulate neurogenesis by increasing the survival of cells in the dentate gyrus in the hippocampus [28]. Because of its regulatory function on

neurogenesis, BDNF appears to be a potential candidate to explore in order to ascertain whether it plays a role in the mechanism of action of LEO to decrease depressive symptoms.

The mechanism of action by which LEO acts to relieve depressive symptoms has not been previously documented. In the present study, serum levels of BDNF, corticosterone and oxytocin were measured 2–3 days after the 14-day treatment period to investigate whether these molecules were implicated in the mechanism of action of LEO. However, caution should be taken when interpreting the results due to the 2–3 days gap between the last administration of the treatments and the collection of the blood samples. The regulatory mechanisms and changes in the concentration of BDNF, corticosterone and oxytocin could have taken place during the 2–3 days gap as observed in corticosterone levels in the Cort group described below. The levels of BDNF were observed 2–3 days after the treatment was stopped showing an increase in the serum concentration of BDNF in the LEO group. The observed results, although not conclusive, suggest that changes in the regulation of BDNF might take place when LEO is administered. However, further studies are needed to draw a clearer picture on the relationship between LEO and serum BDNF. Previous studies have reported that increased BDNF stimulates neurogenesis in the adult hippocampus and SVZ [96]. BDNF plays a key role in the regulation of adult neurogenesis [30,97] and the link between BDNF and depression has been previously established [86,98–101]. Interestingly, the corticosterone group also showed an increase in the BDNF level in serum 2–3 days after the 14-day treatment period was stopped, a situation which might have occurred as a result of the time the serum sample was taken. After 2–3 days of stopping the administration of the treatments, the rats were sacrificed and serum samples were taken. The expression of BDNF in the brain is a regulated mechanism. In a previous study, down-regulation of BDNF mRNA levels was observed after administration of corticosterone [91]. However, 24 h after administration of corticosterone, the expression of BDNF mRNA as well as the BDNF in serum returned to levels comparable to the levels of the control [91]. High levels of corticosterone decreased the excitability of neurons in the hippocampus which led to decreased secretion of BDNF [91]. The increased BDNF level observed 2–3 days after the corticosterone treatment was stopped might be due to potential build-up of BDNF in the neurons as a result of the decreased secretion induced by corticosterone [91]. The BDNF build-up phenomenon after corticosterone treatment may explain the increase in the concentration of BDNF in the Cort group at the time the serum sample was taken.

The corticosterone concentration in serum 2–3 days after stopping the administration of the treatments in the Cort group was low. This was expected after chronic administration of Cort leading to a negative feedback in the HPA axis [67]. Activation of the HPA axis takes place after exposure to stressors, leading to increased levels of circulating glucocorticoids [102]. The HPA axis is subjected to negative feedback which functions as a regulatory mechanism to prevent hormone oversecretion and to maintain the hormonal level within a homeostatic range [103]. Exogenous administration of glucocorticoid leads to suppression of the HPA axis activity which can last several hours and it is reversible at low doses of glucocorticoid [104]. Chronic exogenous administration of corticosterone results in reduced adrenal gland weight which leads to a reduction in the normal endogenous compensatory corticosterone release [105]. In the present study, the exogenous administration of corticosterone was stopped 2–3 days before taking the serum sample. Due to the suppression of the secretion of endogenous corticosterone as a result of the chronic corticosterone administration, the level of corticosterone was low in the Cort group at the time the serum sample was taken. However, the adrenal gland atrophy, negative behavioral changes, and decreased cell proliferation in the hippocampus showed the effect of the chronic administration of corticosterone which has been previously reported [94,102,105,106]. In the LEO group, the corticosterone levels in serum 2–3 days after stopping the administration of the treatment were comparable to those of the control group. Interestingly, in the LEO + Cort group, LEO ameliorated the negative feedback in the HPA axis observed when exogenous corticosterone was administered.

Oxytocin is a hypothalamic neuropeptide produced in the paraventricular and supraoptic nuclei of the hypothalamus. It plays a role in both peripheral (reproduction) and central (social and bonding behavior) processes [107–109]. Attenuation of the behavioral and neuroendocrine effects induced by stress have been observed after oxytocin administration in the dorsal hippocampus [108]. Oxytocin has shown a protective effect against the stress-induced damage to the hippocampus. It is thought to facilitate hippocampal plasticity by increasing cell proliferation and neurogenesis within the hippocampus [32]. The present study shows that the levels of peripheral oxytocin were increased at 2–3 days after stopping the administration of the treatment. However, further studies are needed to understand the relationship between the effects of LEO on oxytocin levels.

5. Conclusions

The present study has demonstrated the potential therapeutic effect of the application of LEO. The observed in vivo effects at both behavioral and cellular level support the use of LEO as a promising treatment option for depression. Not only did LEO improve depression-like behavior but it also promoted neurogenesis and improved dendritic branching. The findings indicate that neurogenesis may play a significant role in the mechanism of action involved when using LEO to treat depression. The changes in the levels of BDNF and oxytocin observed in the LEO group after 2–3 days of stopping the administration of the treatment need further investigation to draw a clearer picture of their role in the behavioral and cellular outcomes under the settings of the present study. Therefore, the evidence from the present study on the upregulation of BDNF and oxytocin is not conclusive. Finally, the results from the present study provide a better understanding of the potential use of LEO for the treatment of depression.

Declaration of interest

None

Statement on welfare of animals

All the procedures involved in the handling of the animals were approved by the Animal Subjects Ethics Sub-Committee at the Hong Kong Polytechnic University.

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