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Estrous cycle affects the neurochemical and neurobehavioral profile of carvacrol-treated female rats

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ABSTRACT

Carvacrol is the major constituent of essential oils from aromatic plants. It showed antimicrobial, anticancer and antioxidant properties. Although it was approved for food use and included in the chemical flavorings list, no indication on its safety has been estimated. Since the use of plant extracts is relatively high among women, aim of this study was to evaluate carvacrol effects on female physiology and endocrine profiles by using female rats in proestrus and diestrus phases.

Serotonin and metabolite tissue content in prefrontal cortex and nucleus accumbens, after carvacrol administration (0.15 and 0.45 g/kg p.o.), was measured. Drug effects in behavioral tests for alterations in motor activity, depression, anxiety-related behaviors and endocrine alterations were also investigated.

While in proestrus carvacrol reduced serotonin and metabolite levels in both brain areas, no effects were observed in diestrus phase. Only in proestrus phase, carvacrol induced a depressive-like behavior in forced swimming test, without accompanying changes in ambulation. The improvement of performance in FST after subchronic treatment with fluoxetine (20 mg/kg) suggested a specific involvement of serotonergic system. No differences were found across the groups with regard to self-grooming behavior. Moreover, in proestrus phase, carvacrol reduced only estradiol levels without binding hypothalamic estradiol receptors. Our study showed an estrous-stage specific effect of carvacrol on depressive behaviors and endocrine parameters, involving serotonergic system.

Given the wide carvacrol use not only as feed additive, but also as cosmetic essence and herbal remedy, our results suggest that an accurate investigation on the effects of its chronic exposure is warranted.

Introduction

In addition to conventional medicine, complementary, alternative and herbal medicines are widely used across the world. In particular, the use of plant extracts is relatively high among women, especially among middle-aged and old women, with higher levels of education and higher incomes, and also with chronic diseases or poor overall health (Brett and Keenan, 2007). Although use of phytotherapy is prevalent in the general population, its use is even more common among women with depression (Eisenberg et al., 1993; Astin, 1998; Eisenberg et al., 1998; Unutzer et al., 2000; Kessler et al., 2001; Wang et al., 2001; Barnes et al., 2004).

At present, no data are available about the content of carvacrol in herbal remedies taken from women to automedicate.

Many reports have documented evidence of the involvement of serotonergic system in the etiology of depression (Coppen et al., 1967; Stockmeier, 2003; Krishnan and Nestler, 2008) and although a dysfunctional serotonin system alone cannot explain the full pathophysiology, it is considered a key factor in depression (Michelsen et al., 2008). In this regard, tryptophan depletion studies confirmed the relationship between serotonin and this psychiatric disorder (Bell et al., 2001; Russo et al., 2009).

Oregano, thyme and majorana are native from the Mediterranean region east to eastern Asia. Several studies have reported that carvacrol (2-methyl-5-(1-methylethyl) phenol; $C_{10}H_{14}O$; mol. wt. 150.21) is the major natural constituent (70%) of essential oils of these aromatic plants (Baser, 2008) and the pharmacological actions of these essential oils are suggested to be parallel to carvacrol content (Aydin et al., 2007; Li et al., 2010).

Despite the large use of these herbal medicines, up to now little is known about the physiological effect of this terpene. Several studies underlined antimicrobial (Periago and Moezelaar, 2001; Kristinsson et al., 2005), antioxidant (Aydin et al., 2005; Horvathova et al., 2006) and anti-genotoxic in vitro activities (Ipek et al., 2005). Essential oils are also of potential interest as an alternative to antibiotic growth promoters, banned in the European Union because of safety issues

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Additionally, several evidences have demonstrated that plant-derived essential oils exhibit a variety of centrally active properties (Sousa et al., 2004; Silva et al., 2007). Indeed, due to their small molecular size and lipophilicity, volatile constituents of essential oils, such as carvacrol, are likely to readily cross the blood–brain barrier (Savelev et al., 2004). In particular, it has been suggested a possible modulatory action of carvacrol in the mammalian Central Nervous System (CNS) (Parnas et al., 2009). Besides, carvacrol seems to possess in vitro acetylcholinesterase inhibitory activity on the CNS (Jukic et al., 2007). However, to the best of our knowledge, no information is available on the effects of carvacrol on female reproductive physiology and endocrine profiles. Estrogen is a well-known regulator of mood, with reported effects of estradiol treatment ranging from depressant to antidepressant properties (Fink et al., 1998; Shors and Leuner, 2003). Furthermore, although many reports have linked estrogens to mood disorders in women, little is known about the role played by phytoestrogens and/or herbal medicines.

In the present study, by using in vivo and ex vivo approaches, we evaluated the neurochemical and behavioral profiles of adult female rats treated with carvacrol. Female rats were analyzed in two different phases of the estrous cycle, diestrus and proestrus, characterized by low and high levels of estrogens, respectively, in order to rule out the influence of gonadal hormones on the action of carvacrol. We evaluated possible alterations on monoaminergic transmission, which controls affective state and emotional responses. In particular, we investigated whether, 2 h after administration, serotonergic neurotransmission in rat prefrontal cortex (PFC) and nucleus accumbens (NAc) were affected by oral carvacrol treatment. Additionally, this study was designed to evaluate the effects of carvacrol in different behavioral tests to investigate for alterations in motor activity, depression and anxiety-related behaviors by using the open field and the forced swimming test. Finally, carvacrol-induced endocrine alterations were investigated in the plasma of female rats.

Methods

Animals. Adult female Wistar rats (Harlan, S. Pietro al Natisone, UD, Italy) weighing 200–250 g were used. The animals were randomly assigned to the experimental groups (n = 4–10 per group), one for each behavioral, neurochemical and biochemical analysis and they were allowed to acclimatize to the animal house for at least 7 days before the experiments. They were housed in standard cages in a controlled temperature room (22 ± 1 °C), and relative humidity (55 ± 5%) under a 12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM). Standard laboratory chow and tap water were available ad libitum. The experiments were conducted in accordance with guidelines released by Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B), and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (NIH Publications No. 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

Experimental protocol. After a week of habituation to housing conditions, female rats were subjected daily for two weeks to vaginal smears. Vaginal samples were taken every morning between 8:00 and 9:00 AM using a cotton stick inserted into the vagina not deeply. Then, vaginal fluid was placed on a glass slide and fixed with "Diff-Quick Staining Protocol". It consists in fixing in "Diff Quick" Fixative (Solution I) for 10 s, staining with "Diff Quick" (Solution II, pink) for 10 s and, finally, counterstaining with "Diff Quick" (Solution III, blue) for 8 s. Then, each slide was rinsed in water to remove excess stain and, when dry, observed under a light microscope, with 40× objective lenses.

The reproductive cycle of female rats is characterized by four phases: proestrus, estrus, diestrus and metestrus. The predominance of nucleated epithelial cells represented the proestrus phase, while the diestrus smear primarily consisted of a predominance of leukocytes with the presence of sporadic epithelial cells (Marcondes et al., 2002).

Only regularly cycling rats (estrous cycle: 4–5 days) were assigned to experimental group, namely proestrus and diestrus, according to vaginal smear results obtained, on the test day.

Carvacrol (0.15 or 0.45 g/kg) or vehicle (peanut oil, 1 ml/kg) was orally administered in the morning immediately after vaginal cytology procedure. All experimental analyses were carried out before 2:00 PM, 2 h after carvacrol administration to avoid the influence of endocrine changes. Indeed during the estrous cycle, prolactin, LH and FSH remain low and increase in the afternoon of the proestrus phase (Marcondes et al., 2002).

Moreover, considering that the estrous cycle is very short, the association between carvacrol administration and changes in function was verified also at the end of each experiment, through the re-characterization of the vaginal smear.

Chemicals. Carvacrol (purity>98%), fluoxetine (20 mg/kg s.c. dissolved in deionized water) and unlabelled estradiol were purchased from Sigma Aldrich s.r.l. (Milan, Italy). The drug was dissolved in peanut oil and administered per os, by gavage, at two different doses (0.15 g/kg and 0.45 g/kg). Carvacrol and vehicle were administered in a volume of 1 ml/kg. Diff Quick Staining Solutions were purchased from Dade Behring (Milan, Italy). ^{3}H-estradiol ((Silva et al., 2007,16,17-3H-(N)) estradiol) was purchased from PerkinElmer, Waltham, MA, USA.

Plasma sample collection. Trunk blood was collected from naïve or treated female rats in proestrus and diestrus phases using heparinised tubes. Samples were centrifuged at 10,000 ×*g* for 20 min. at 4 °C. Supernatants were removed and frozen at −80 °C until analyses.

Progesterone quantification. Progesterone levels were measured by ELISA using commercially available kit (USCN Life Science Inc., Wuhan, China) according to manufacturer's instructions. Briefly, colorimetric detection of peroxidase activity was achieved by adding TMB solution and incubating for 15 min at 37 °C according to the manufacturer's instructions. The enzymatic reaction was stopped with Stop Solution and the optical density of each well was measured at 450 nm using a PowerWave XS plate reader (Bio-Tek, Winooski, VT, USA). Each analysis was performed in duplicate in the same assay to avoid inter-assay variations. Progesterone levels are expressed as ng/ml.

Estradiol quantification. Estradiol levels were measured by ELISA using commercially available kit (Cayman Chemical, Ann Arbor, USA) according to manufacturer's instructions. The assay was based on the competition between estradiol and an estradiol-acetylcholinesterase conjugate for estradiol antiserum. Optical density of each well was measured at 405 nm using a PowerWave XS plate reader (Bio-Tek, Winooski, VT, USA). Each analysis was performed in duplicate in the same assay to avoid inter-assay variations. Estradiol levels are expressed as pg/ml.

Post-mortem tissue analyses. Rats were killed by decapitation 2 h after drug administration and brains were immediately removed. For dissection, the brains were placed dorsal side up in an ice chilled rat brain matrix (World Precision Instruments, Inc. FL, USA) with slits spaced at 1 mm using an ice-chilled razor blade. Target regions were dissected out and weighted, according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Thereafter, PFC and NAc were