

EFFECTS OF CHLOROGENIC ACID ON BEHAVIOR AND METABOLISM IN OVARIECTOMIZED RATS

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Abstract

The objective of the present study was to make a complex evaluation of the effect of chlorogenic acid (CGA) on behaviour, lipid metabolism and bone mineral density (BMD) in an ovariectomized (OVX) rat model. Female Wistar rats were divided in 3 groups, each of 14 animals – sham operated (SO), OVX and the last group ovariectomized rats treated with CGA. Three months after the operation, rat behaviour was investigated in the open field test (OFT), elevated plus-maze test (EPM), social interaction test (SIT), forced swimming test (FST) and hot plate test (HPT). Weight gain, total and retroperitoneal fat deposits were measured, as well as serum concentrations of total cholesterol. Femur BMD was also evaluated. CGA managed to improve some of the negative consequences of the oestrogen deficit (anxiety and depressive behaviour, increased pain sensitivity and decreased BMD) and did not affect others (increased fat accumulation and elevated cholesterol levels).

Rezumat

Obiectivul prezentului studiu a fost evaluarea efectului acidului clorogenic (CGA) asupra comportamentului, metabolismului lipidic și a densității minerale osoase (DMO) într-un model de șobolan ovariectomizat (OVX). Femelele de șobolan Wistar au fost împărțite în 3 grupuri, 14 animale *per* lot: operate simulat (SO), ovariectomizate (OVX) și tratate cu acid clorogenic (CGA). La trei luni după operație, comportamentul animalelor a fost investigat prin testul în câmp deschis, testul labirintului, testul de interacțiune socială, testul înotului forțat și testul de sensibilitate pe placă fierbinte. Au fost evaluate creșterea în greutate, depozitele de grăsime totale și retroperitoneale, precum și concentrațiile serice ale colesterolului total. De asemenea, a fost evaluată DMO femurală. CGA a reușit să amelioreze unele dintre consecințele negative ale deficitului de estrogeni (anxietate și comportament depresiv, sensibilitate crescută la durere și scăderea DMO) și nu a prezentat efect asupra altor parametri evaluați (acumulare crescută de grăsime și nivelurile crescute de colesterol).

Keywords: chlorogenic acid, ovariectomy, rats

Introduction

The ovariectomized (OVX) rat is an animal model which simulates the clinical aspects of menopause [18]. The postmenopausal decline in ovarian function often leads to dyslipidaemia (decreased HDL levels and increased total cholesterol and LDL) and increased bone turnover with subsequent osteoporosis and increased fracture risk [23]. Menopause is also linked to a greater risk of obesity and increased abdominal fat [21]. There is some association between menopause and depression, although it is more likely that the depressive state is related to menopausal symptoms rather than to oestrogen levels [2]. In animal models, menopause is associated with a decreased pain sensitivity threshold [27].

Chlorogenic acid (CGA) is a phenolic acid present in natural food sources such as coffee, tea, apples,

pears, plums, chokeberry [28] etc. The main source of CGA in the European diet is coffee [8]. The aim of the present study was to make a complex evaluation of the effects of CGA on different aspects of OVX-induced oestrogen deficit in rats.

Materials and Methods

Forty-two sexually naive four-months old female Wistar rats were divided in 3 groups – OVX (ovariectomized rats), SO (sham operated rats) and CGA (ovariectomized rats treated with CGA). Before surgery, rats underwent general anaesthesia using a combination of ketamine 30 mg/kg bw and xylazine 30 mg/kg bw. Animals were fixed, the abdominal hair was removed and the skin was disinfected using iodine. The abdominal cavity was opened by a midline incision. In the SO groups rats were sewed back

immediately. In OVX and CGA rats, ovaries were isolated, fallopian tubes were clamped and a thread was tightly tied around the oviduct including blood vessels. After closing of the abdominal wall, animals were prone to postoperative antibiotic prophylaxis with 200 mg/kg bw Cefazolin i.p. Rats underwent a two-week postoperative recovery period. Animals were housed in plastic cages in a ventilated room maintained at $22 \pm 1^\circ\text{C}$ and on a 12/12 light/dark cycle. SO and OVX rats were treated with distilled water 10 mL/kg bw. CGA was prepared as a 2 mg/mL aqueous solution. From this solution, CGA-treated rats received 10 mL/kg bw which gave a dose of 20 mg/kg bw CGA. Every week until the end of the experiment, animal weight was measured. Three months afterwards, on different days, several behavioural tests were performed: open field test (OFT), elevated plus maze test (EPM), social interaction test (SIT), forced swim test (FST) and hot plate test (HPT). At the end of the experiment, the animals were anesthetized with diethyl ether. Blood from the sublingual veins was collected and serum was prepared for biochemical investigations. Retroperitoneal and total body fat deposits were measured. Femurs were also taken for analysis. All experimental procedures were conducted according to the national laws and policies, and in conformity with the international guidelines (EU Directive, 2010/63/EU for animal experiments). The experiments were approved by the Bulgarian Food Safety Agency (document № 148/ 04.08.2016).

Open field test (OFT)

OFT took place in a square arena 100 x 100 x 40 cm. The floor of the box was divided by lines into 25 equal squares. Rats were put one at a time in the centre of the box and their behaviour was closely watched for 5 minutes. The line crossings (horizontal movements) and rearings (vertical movements) were used as a measure of locomotor activity while the time spent in the centre (central 9 squares) was a measure of anxiety [15].

Elevated plus maze (EPM) test

EPM was carried out in an X-maze with two open and two closed arms elevated at 50 cm above the floor. Rat behaviour was observed for 5 min. The rat was put in the centre of the maze and the number of open-, closed- and total arm entries was detected, as well as the time spent in the open and in the closed arms. The index of open- vs. total arm entries was calculated as well. In this test, anxiety levels in rodents are considered inversely proportional to the number of open-arm entries and the time spent there [24].

Social interaction test (SIT)

SIT was performed in the same square arena used for OFT. Two unfamiliar rats with similar weights were released in the opposite angles of the box. Their behaviour was recorded for 5 min and the time spent in social interaction (sniffing, following, wrestling

and crawling under or over the other rat) was measured. This test is used to assess anxiety in rodents, as levels of anxiety are inversely proportional to the time spent in interaction [11].

Forced swim test (FST)

The forced swimming test (FST), called also Porsolt test, is widely used to assess behavioural despair in rodents. It was performed in a glass cylinder filled with water. The rodent was put inside it for 5 min and was thus forced to swim. There was a training session and on the next day immobility time was measured as a marker of depressive behaviour. After swimming, animals were wiped and dried before returning to their home cages [25].

Hot plate test (HPT)

HPT is widely used to encounter effects on thermal nociception in rodents. It was carried out on a heated (52°C) surface enclosed by a glass cylinder with a diameter of 24 cm (Ugo Basile S.R.L., Italy). Time latency before shaking or licking the paw or before jumping was measured and taken as an index for nociceptive pain sensitivity. Animals were removed from the plate immediately after responding or after a cut-off time (45 sec) to prevent tissue damage. Three consecutive measures at an interval of 2 hours were performed and the mean value was calculated for each animal.

Biochemical measurements

Serum levels of total cholesterol were measured spectrophotometrically (spectrophotometer CE2021, Cecil Instruments Ltd, UK). The analysis was performed using kits from Biomaxima (Poland) according to manufacturer's instructions.

X-ray absorptiometry (DXA)

After removing all soft tissues from the femurs they were frozen until the bone mineral density (BMD) was measured by dual energy X-ray absorptiometry (DXA) using a computer program for small subjects.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 5 computer program using two-tailed t-test. Data are presented as mean \pm SEM. A value of $p < 0.05$ was considered significant.

Results and Discussion

Rat behaviour

In the OFT, the number of horizontal and vertical movements of OVX rats were decreased slightly, but not significantly in comparison with SO animals. CGA administration decreased the number of crossings and rearings in comparison with SO group ($p < 0.05$), but did not change them significantly if compared to OVX group. There was no significant difference between the times spent in the central quadrants (sec) in the three groups, although the longest central time was observed in the CGA group (Table I).

The results from the EPM test are presented in Table II. In the EPM test, there was no statistically significant difference between OVX and SO groups concerning the number of entries into the open and closed arms of the maze, total number of arm entries, index of open/total arm entries, as well as time spent in the

open and closed arms. CGA was able to decrease the number of entries into the closed arms, as well as the total number of arm entries ($p < 0.01$ for both indices) in comparison with SO, but not with OVX animals. It did not change significantly other indices recorded.

Table I

Number of crossings and rearings and time spent in the central quadrants (central time) in the OFT; time spent in social interaction (SI) in SIT and immobility time in FST

Groups of animals	Test results	Crossings	Rearings	Central time (sec)	Time for SI (sec)	Immobility time (sec)
SO		56.1 ± 10.3	9.5 ± 1.6	2.6 ± 0.6	30.4 ± 4.6	54.7 ± 6.8
OVX		40.9 ± 5.7	7.4 ± 0.8	2.9 ± 0.4	12.3 ± 1.9***	85.8 ± 8.4**
CGA		23.9 ± 6.6*	5.1 ± 0.8*	3.8 ± 0.6	22.1 ± 5.3	79.9 ± 9.9*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SO

Table II

Number of open arm (OA), closed arm (CA) and total arm (TA) entries, index of open vs. total arm entries, time spent in the open and closed arms (sec) in the elevated plus maze test

Groups of animals	Test results	OA entries	CA entries	TA entries	OA/TA entries	Time in OA (sec)	Time in CA (sec)
SO		2.1 ± 0.5	4.7 ± 0.6	6.8 ± 0.9	0.3 ± 0.1	30.3 ± 6.7	269.7 ± 6.6
OVX		2.1 ± 0.4	3.1 ± 0.7	5.2 ± 1.0	0.4 ± 0.1	35.6 ± 8.4	264.4 ± 8.4
CGA		1.8 ± 0.3	2.1 ± 0.4**	3.9 ± 0.5**	0.5 ± 0.1	24.9 ± 4.1	275.1 ± 4.1

** $p < 0.01$ vs. SO

In the SIT, the time spent in social interaction was significantly decreased ($p < 0.001$) in the OVX group in comparison with SO group. CGA was not able to contribute to any significant change neither compared to SO, nor to OVX rats, although the time for SI of CGA - treated animals was longer than that of OVX rats (Table I).

In the FST, the immobility time of OVX rats ($p < 0.01$) and of CGA-treated rats ($p < 0.05$) was significantly prolonged compared to that of SO rats. Although there was a small decrease when compared to OVX animals, CGA did not change the immobility time significantly (Table I).

In the HPT, the time latency was significantly decreased ($p < 0.05$) in the OVX group compared to SO group (28.3 ± 1.2 vs. 32.6 ± 1.4 sec). CGA prolonged the latency (34.7 ± 1.1 sec) when compared to OVX rats ($p < 0.001$). There was no significant difference between SO and CGA groups.

Lipid metabolism

Total as well as retroperitoneal fat deposits of the OVX rats were significantly ($p < 0.01$) higher than those of SO animals. The indices of total fat/animal weight ($p < 0.05$) and retroperitoneal fat/animal weight ($p < 0.01$) were also significantly increased in OVX

vs. SO group. In the CGA group, fat deposits were not significantly changed in comparison with OVX animals, but were significantly higher if compared to SO rats ($p < 0.001$ for total and retroperitoneal fat, $p < 0.01$ for total fat/weight index and $p < 0.001$ for retroperitoneal fat/weight index).

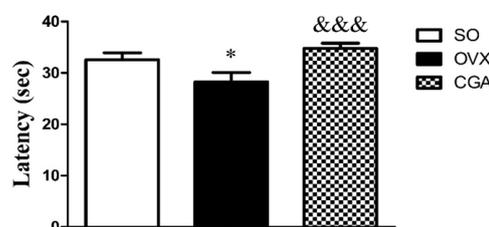


Figure 1.

Latency in HPT (sec); SO – sham-operated rats; OVX – ovariectomized rats; CGA – rats treated with chlorogenic acid

* $p < 0.05$ vs. SO; *** $p < 0.001$ vs. OVX

Total cholesterol in serum was significantly elevated ($p < 0.05$) in OVX group in comparison with SO group. CGA - treated animals did not show significant change in cholesterol levels neither compared to SO, nor to OVX group.

Table III

Lipid metabolism. Total (TF) and retroperitoneal (RPF) fat, indices of total fat/weight and retroperitoneal fat/weight and total plasma cholesterol (TC) (mmol/L)

Groups of animals	Test results	TF (g)	RPF (g)	TF/weight	RPF/weight	TC (mmol/L)
SO		9.4 ± 1.2	1.6 ± 0.2	0.04 ± 0.005	0.006 ± 0.001	2.1 ± 0.1
OVX		15.4 ± 1.8**	3.3 ± 0.4**	0.05 ± 0.005*	0.012 ± 0.001**	2.4 ± 0.1*
CGA		16.3 ± 1.4***	3.9 ± 0.4***	0.06 ± 0.004**	0.015 ± 0.001***	2.3 ± 0.1

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SO

Bone mineral density (BMD)

Femur BMD was slightly, but not significantly decreased in OVX vs. SO group (0.263 ± 0.002 vs. 0.273 ± 0.014 g/cm²). In the CGA group BMD was increased (0.291 ± 0.008 g/cm², $p < 0.05$) in comparison to OVX rats and not significantly different from that of SO rats.

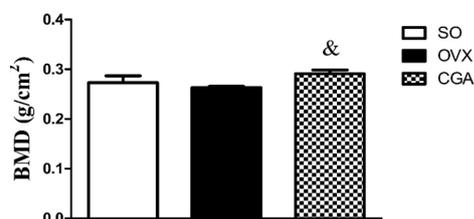


Figure 2.

Femur bone mineral density (BMD); SO – sham-operated rats; OVX – ovariectomized rats; CGA – rats treated with chlorogenic acid
& $p < 0.05$ vs. OVX

Oestrogens are one of the key players in the modulation of bone metabolism [6]. A possible mechanism by which they exert bone protection is by up-regulating osteoblastic Fas-ligand expression resulting in apoptosis of osteoclast precursors [19]. The ovariectomized (OVX) rat is a golden standard model in order to mimic the changes in female organism during menopause [18]. Usually the duration of the model is three months or 100 days [10], although shorter or longer interventions are also present in literature. Usually, studies investigate separately either the behavioural effects of oestrogen deficit [29] or the changes in BMD and/or lipid metabolism [5, 26]. There are some data concerning the positive effect of CGA on OVX-induced osteoporosis [12, 22]. This experiment aimed to elucidate the complex effects of CGA on behaviour, lipid metabolism and BMD of OVX rats. OVX and CGA treated animals showed a decreased locomotor activity (decreased number of horizontal and vertical movements in OFT and decreased number of closed- and total arm entries in EPM test) in comparison with SO animals (no statistical significance for OVX vs. SO, significant for CGA vs. SO). This decrease in the locomotor activity might be due to a sedative effect of CGA, demonstrated in previous studies with CGA in healthy animals [14]. The possible mechanism of this sedative action is probably linked to GABA-receptor activation [16].

SIT showed an increased anxiety in OVX rats compared to SO animals (indicated by the decreased time for social interaction). In FST, OVX animals showed an increased immobility time which indicated the development of depression. Such findings are typical for the OVX model of oestrogen deficit [9, 29]. CGA was able to prolong the time for social interaction and decrease the immobility time in FST in comparison with OVX animals. These findings showed no statistical significance, but could still

indicate that CGA prevented OVX-induced anxiety and depression to a certain extent. The anxiolytic effect might be due to activation of BDZ-receptors [4].

In HPT, OVX animals showed thermal hyperalgesia as expected [20, 27]. Polyphenols have been effective antinociceptive agents in animal models of pain [1]. CGA decreased thermal hyperalgesia of OVX rats. The antioxidant, anti-inflammatory, neuroprotective and neurotrophic action of CGA possibly all contributed to its antidepressive effect [13] and to the reversal of OVX-induced hyperalgesia [3].

Increased total and retroperitoneal fat deposits were observed in OVX animals, which resulted in elevated indices of total fat/animal weight and retroperitoneal fat/animal weight, respectively. Plasma total cholesterol levels in OVX animals were also increased. These findings are also typical for the OVX rat model [26]. There are literature data showing that CGA treatment decreased body weight and fat in male mice with high-fat-diet induced obesity [7]. One of the aims of this study was to find out whether CGA would be able to reverse the fat accumulation resulting from the oestrogen deficit. The results showed that CGA did not antagonize fat accumulation and plasma cholesterol elevation in OVX rats. This might be due to the fact that the regulation of hepatic lipid-metabolism related enzymes by CGA was not sufficient to induce weight loss in conditions of oestrogen deficiency [30] and CGA did not demonstrate an estrogenic activity [17]. In our experiment, the BMD of OVX rats were slightly decreased (no statistical significance). CGA showed a significant increase of BMD in OVX rats possibly by increased proliferation and differentiation of osteoblastic precursors [31] and also by inhibition of apoptosis and promotion of bone remodelling by MAPK pathway [22].

Conclusions

CGA prevented to a certain extent the negative effects of OVX-induced oestrogen deficit on anxiety and depressive behaviour as well as on BMD. CGA did not improve the changes in lipid metabolism caused by the oestrogen deficit.

Conflict of interest

The authors declare no conflict of interest.

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