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Original Article

Effects of Ovariectomy-Induced Estrogen Deficit on Rat Behaviour, Lipid Metabolism, Inflammation, Bone Mineral Density, and Turnover

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Abstract

Aim: The objective of the present study was to make a complex evaluation of behaviour, lipid metabolism, inflammation, and bone turnover in an ovariectomized rat model used to simulate postmenopausal clinical findings.

Materials and methods: Female Wistar rats were divided into 2 groups of 16 animals each: sham operated (SO) animals and ovariectomized (OVX) animals. Three months after the operation, a battery of behavioural tests was performed including an open field test (OFT), elevated pus-maze test (EPM), the social interaction test (SIT), the forced swim test (FST), and a hot plate test (HPT). At termination of experiment, weight gain and fat deposits (total and retroperitoneal) were measured. Serum concentrations of blood lipids were determined. Tumor necrosis factor alpha (TNF-alpha) and alkaline phosphatase (ALP) serum concentrations were used for evaluation of the inflammation and bone turnover, respectively. Femur bone mineral density (BMD) was evaluated using dual energy X-ray absorptiometry.

Results: OVX rats did not demonstrate any significant behavioural changes in OFT and EPM tests but showed a decreased interaction time in SIT and an increased immobility time in FST test which indicated anxiety and depression. The OVX rats had a significantly lower pain sensitivity threshold. They had greater weight gain, increased total and retroperitoneal fat deposits, as well as elevated total fat/body weight and retroperitoneal fat/body weight ratios. Blood cholesterol, ALP and TNF-alpha of the OVX group were also significantly higher. Femur BMD of OVX rats was slightly but not significantly reduced.

Conclusions: Estrogen deficiency in OVX rats caused depression, anxiety, hyperalgesia, obesity, dyslipidemia, and inflammation before the reduction in bone mineral density was prominent.

Keywords

anxiety, depression, obesity, ovariectomy, rats

INTRODUCTION

The ovariectomized (OVX) rat is an animal model which simulates the clinical findings in postmenopausal conditions.[1] In menopause, estrogen levels are decreased because of decline in ovarian function. This often leads to dyslipidemia (decreased HDL levels and increased total cholesterol and LDL) and increased risk of ischemic heart disease, increased bone turnover with subsequent osteoporosis and increased fracture risk, and some decline in cognitive function.[2] Menopause is also linked to a greater risk of obesity and increased abdominal fat[3], accompanied by a low grade inflammation[4]. There is some association of menopause with depression, although it is more likely that the depressive state is related to menopausal symptoms rather than to estrogen levels.[5] In animal models, menopause presents with decreased pain sensitivity threshold.[6]

AIM

The aim of the present study was to make a complex evaluation of many aspects of behaviour, metabolism, inflammation, bone turnover and bone mineral density in a rat model of ovariectomy-induced estrogen deficit.

MATERIALS AND METHODS

Thirty-two sexually naive, 4-month old female Wistar rats were divided into 2 groups – ovariectomized (OVX) and sham-operated (SO). Before surgery, rats underwent general anesthesia using a combination of ketamine (30 mg/kg) and xylazine (30 mg/kg). Animals were fixed, abdominal hair was removed and skin was disinfected using iodine. The abdominal cavity was opened by a midline incision. In the SO rats, it was sewed back immediately. In the OVX rats, ovaries were isolated, fallopian tubes were clamped and a thread was tightly tied around the oviduct including blood vessels. After closing the abdominal wall, a single antibacterial prophylactic dose of cefazolin (200 mg/kg) was administered intraperitoneally. The rats underwent a two-week postoperative recovery period. The animals were housed in plastic cages in a well-ventilated room maintained at 22±1°C and on a 12/12 light/dark cycle. The animals' weights were measured once weekly until the end of the experiment. Three months afterwards, on different days, a behavioural test battery was performed which included the open field test (OFT), the elevated plus maze (EPM), the social interaction test (SIT), the forced swim test (FST), and the hot plate test (HPT). At the end of the experiment, the animals were anesthetized with diethyl ether. Blood was collected from sublingual veins 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, in conformity with the international guidelines (EU Directive, 2010/63/EU for animal experiments).

Open field test (OFT)

OFT took place in a square arena 100×100×40 cm. The floor of the box was divided by lines into 25 equal squares. Rats were put, one at a time, in the center of the box and their behaviour was closely watched for 5 minutes. The line crossings (horizontal movements) and rearings (vertical movements) were taken as a measure of locomotor activity and exploratory behaviour while the time spent in the center (central 9 squares) was a measure of anxiety.[7]

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 center of the maze and the number of open and closed arms and the total arm entries was recorded, as well as time spent in the open and in the closed arms. The index of open- vs. total arm entries was calculated as well. This behavioural test gives information about the level of anxiety in rodents because it is their natural behaviour to seek safety by preferring the closed arms of the maze. Anxiety levels are inversely proportional to the number of open-arm entries and the time spent there.[8]

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 corners of the box. Their behaviour was recorded for 5 min and the time spent in social interaction was measured. Sniffing, following, wrestling, crawling under or over the other rat were considered an interaction, while passive contact such as lying or sitting over, under or next to the other animal were not. This test is used to assess anxiety in rodents, as levels of anxiety are inversely proportional to the time spent in interaction.[9]

Forced swim test (FST)

The forced swim 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, the animals were wiped and dried before they returned to their home cages.[10]

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 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, triglycerides and alkaline phosphatase (ALP) were measured spectrophotometrically (spectrophotometer CE2021, Cecil Instruments Ltd, UK). TNF-alpha was measured by ELISA microplate reader 800 TS, Bio Tec Instruments, Inc. (USA). The biochemical analyses were performed using commercial kits according to manufacturers' instructions. Total cholesterol kits were purchased from Biomaxima (Poland), TNF-alpha kits – from Boster Biological Technology (USA), and ALP kits – from Biosystems (Spain).

Dual energy X-ray absorptiometry (DXA)

After removing all soft tissues from the femurs, 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 using GraphPad Prism 5 computer program using two-tailed t-test. Data were presented as mean ± SEM. A value of $p<0.05$ was considered significant.

RESULTS

Rat behaviour

In the OFT, the number of horizontal and vertical movements of OVX rats decreased slightly but not significantly in comparison with SO animals (40.9±5.7 vs. 56.1±10.3 for crossings and 7.4±0.8 vs. 9.5±1.6 for rearings). The time spent in the central quadrants (in seconds) was slightly increased with no statistical significance (2.88±0.43 vs. 2.64±0.61).

In the EPM test, there was no statistically significant difference between OVX and SO groups concerning the number of entries into the open arms of the maze (2.06±0.38 vs. 2.07±0.45), number of entries into the closed arms (3.13±0.69 vs. 4.71±0.63), total number of arm entries (5.19±0.96 vs. 6.79±0.87), index of open/total arm entries (0.40±0.06 vs. 0.31±0.05), time spent in the open arms (35.6±8.4 vs. 30.3±6.7 s) as well as time spent in the closed arms (264.4±8.4 vs. 269.7±6.6 s).

In the SIT, the time spent in social interaction significantly decreased ($p<0.001$) in the OVX group in comparison with SO group (12.3±1.9 vs. 30.4±4.6 s) **(Fig. 1A)**.

In the FST, the immobility time of OVX rats significantly increased ($p<0.01$) compared to that of SO rats (85.8±8.4 vs. 54.7±6.8 s) **(Fig. 1B)**.

In the HPT, the time latency significantly decreased ($p<0.05$) in the OVX group compared to SO group (28.3±1.2 vs. 32.6±1.4 s) **(Fig. 1C)**.

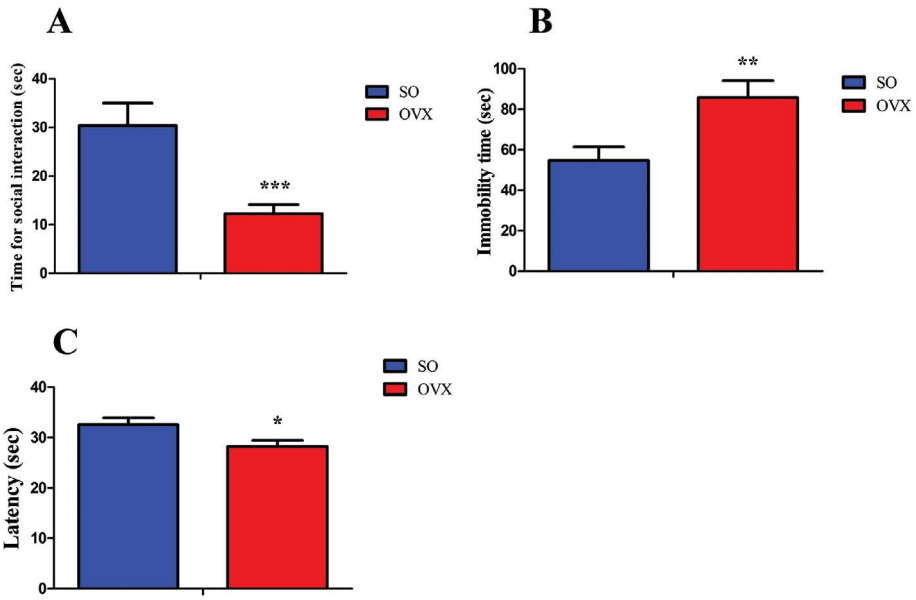

Figure 1. Time spent in social interaction (seconds) in the SIT (panel **A**); Immobility time (seconds) in the FST (panel **B**); Time latency (seconds) before behavioural reaction to pain in the HPT (panel **C**). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; SO: sham-operated; OVX: ovariectomized.

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Lipid metabolism

Total as well as retroperitoneal fat deposits of OVX rats were significantly (p<0.01) higher than those of SO animals (15.39±1.75 vs. 9.35±1.17 g for total fat and 3.34±0.41 vs. 1.58±0.24 g for retroperitoneal fat) **(Figs 2A, 2B)**. The indices of total fat/animal weight (0.054±0.005 vs. 0.036±0.005; p<0.05) and retroperitoneal fat/animal weight (0.012±0.001 vs. 0.006±0.001; p<0.01) were also significantly increased in OVX vs. SO group **(Figs 2C, 2D)**.

Total cholesterol in serum was significantly elevated (p<0.05) in OVX group in comparison with SO group (1.36±0.06 vs. 1.06±0.10 mmol/l) **(Fig. 2E)**.

Inflammation

Serum levels of TNF-α were also significantly elevated (p<0.05) in OVX vs. SO group (52.29±4.10 vs. 36.30±5.98 pg/ml) **(Fig. 3A)**.

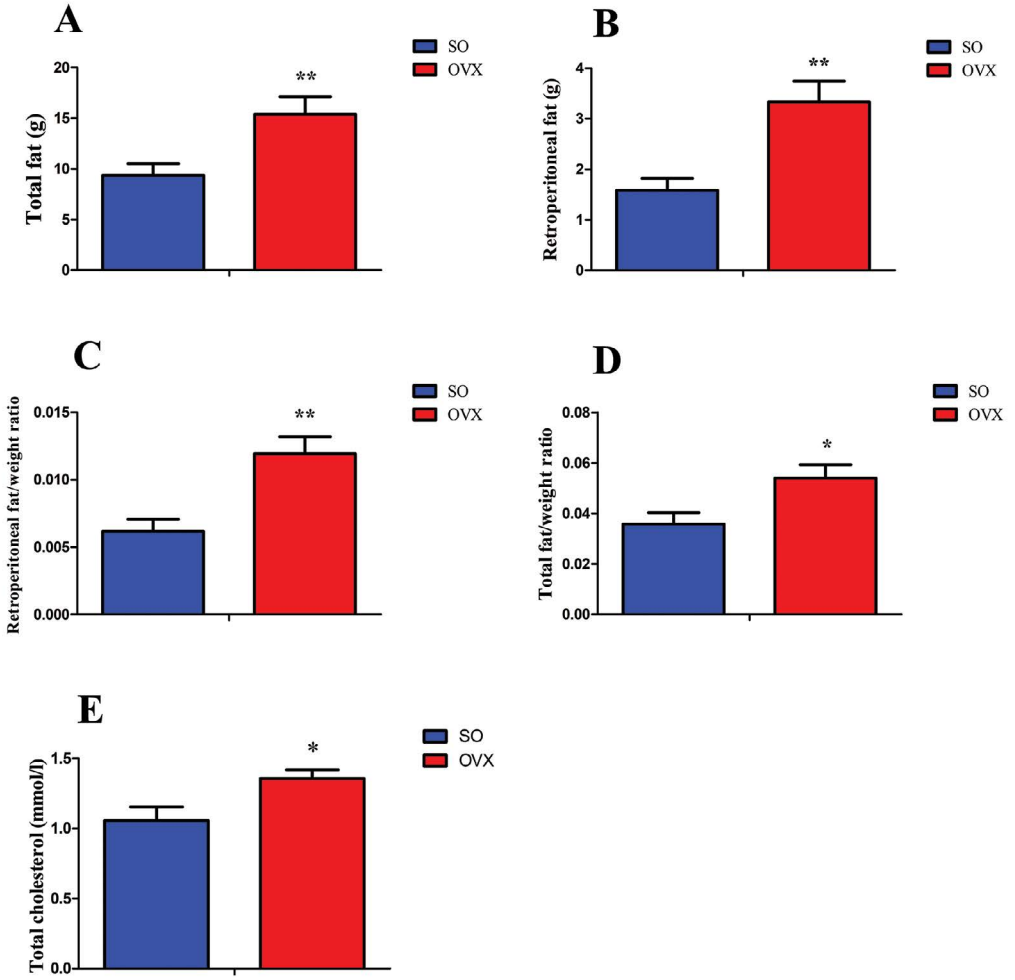

Figure 2. Total fat (panel **A**); Retroperitoneal fat (panel **B**); Retroperitoneal fat/weight ratio (panel **C**); Total fat/weight ratio (panel **D**); Total cholesterol in serum (panel **E**); * p<0.05; ** p<0.01; SO: sham-operated; OVX: ovariectomized.

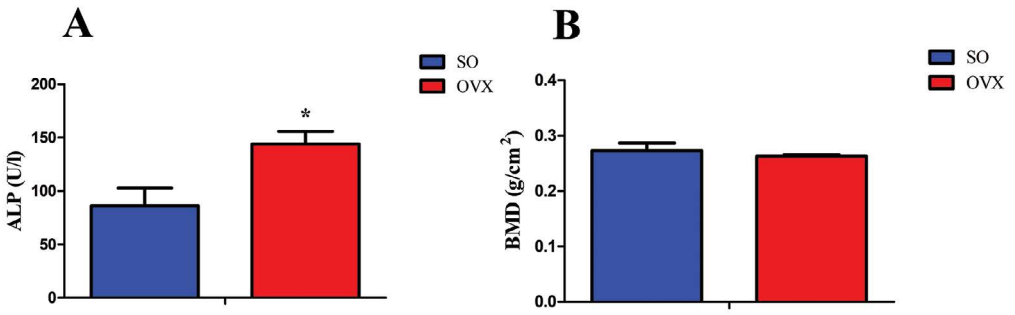

Figure 3. Serum levels of ALP (panel **A**); Femur BMD (panel **B**); * p<0.05; SO: sham-operated; OVX: ovariectomized.

Bone turnover and bone mineral density (BMD)

Serum ALP levels were significantly elevated ($p<0.05$) in OVX group in comparison with SO group (143.9±11.9 vs. 86.5±16.6 U/l) **(Fig. 3A)**. Femur BMD was slightly but not significantly decreased in OVX vs. SO group (0.263±0.002 vs. 0.273±0.014 g/cm^2) **(Fig. 3B)**.

DISCUSSION

Estrogens are key bone metabolic modulators.[11] A possible mechanism by which estrogens exert bone protection is by up-regulating osteoblastic Fas-ligand expression resulting in apoptosis of osteoclast precursors.[12] Ovariectomized rat is the standard gold model in mimicking the changes which women undergo during menopause.[1] Usually, the duration of the model is three months or 100 days[13], although shorter[14] or longer interventions[15] have also been reported in literature. Most researchers prefer to focus either on the behavioural effects of ovariectomy[16-20] or on the changes in bone mineral density, state of inflammation and/or lipid metabolism[13,15,21-28]. This experiment focuses on the complex effects of estrogen deficit on rat behaviour, lipid metabolism, inflammation, bone mineral density and turnover.

In an experiment of Patki et al.[14], OVX rats showed increased levels of anxiety in the OFT three weeks after surgery. De Chaves et al.[16] also observed increased anxiety in the EPM test three months after ovariectomy. In both experiments Wistar rats were used. In this experiment, the shortened social interaction time of OVX animals indicated increased levels of anxiety although in the EPM there was no statistically significant elevation in rat anxiety-like behaviour.

In the FST, OVX rats showed an increased immobility time which was not due to decreased locomotion as demonstrated in the OFT test. This finding indicated the development of depression in OVX rats. Walf et al.[17] and Li et al.[18] have observed similar results 1 month and 6 months after surgery, respectively. It is interesting that de Chaves et al.[16], using the same duration of the experiment (3 months) and the same breed (Wistar rats), found that ovariectomy did not affect immobility time.

The HPT showed that OVX rats exhibited thermal hyperalgesia. This finding is consistent with some other experiments and inconsistent with others. Using the tail-flick test, Li et al.[18] demonstrated that OVX rats developed thermal hyperalgesia beginning from the second week after surgery. In ovariectomized mice, mechanical but not thermal hyperalgesia was present.[19] In a rat OVX model, Hosseini et al.[20] did not find a statistically significant difference between OVX and sham-operated young Wistar rats 6 weeks after surgery.

As far as body composition and lipid metabolism are concerned, we found a significant increase in the total and retroperitoneal fat, total fat/weight and retroperitoneal fat/weight ratio, as well as in serum total cholesterol of OVX rats. Such findings are usual for this animal model.[21-24]

Increased levels of inflammation and bone turnover are also usual in OVX rodents. In this experiment, inflammation was evaluated by determining TNFalpha in blood serum, and bone turnover – by ALP levels. Both were significantly increased, which coincides with other researchers' findings.[25,26]

Our experiment showed that three months after ovariectomy, the femur BMD in OVX rats was slightly but not significantly decreased. This finding is consistent with other results in literature.[13,27] Jiang et al.[27] showed that femur BMD of OVX rats three months after the operation was not significantly reduced and continued to decrease to significant levels six months after the operation. However, other experiments performed on Sprague-Dawley rats[26,28] demonstrated a significant reduction of BMD three months after ovariectomy. In our experimental setting, three months was not enough time for the changes in BMD to become prominent but increased bone turnover was recorded by the statistically significant elevation of ALP.

CONCLUSIONS

This study demonstrates that three months after ovariectomy, Wistar rats show increased levels of anxiety and depression, and decreased pain threshold. These are accompanied by typical changes in lipid metabolism, inflammation and bone turnover, but not in BMD. The value of our experiment lies in the parallel assessment of changes in behaviour, metabolism and BMD, and in the demonstration that behavioural and metabolic changes precede the prominent decrease of BMD in conditions of estrogen deficit.

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Влияние дефицита эстрогена, вызванного овариэктомией, на поведение, липидный метаболизм, воспаление, минеральную плотность костей и обмен веществ у крыс

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Резюме

Цель: Цель настоящего исследования состояла в том, чтобы предоставить всестороннюю оценку поведения, метаболизма липидов и метаболизма костей модели овариэктомии у крыс, используемой для имитации клинических данных в постменопаузе.

Материалы и методы: Самки крыс линии Wistar были разделены на две группы по 8 крыс в каждой: моделируемые (CO) животные и овариэктомия (OVX). Через три месяца после операции был проведен ряд поведенческих тестов, включая тест открытого поля (open field test – OFT), тест приподнятого крестообразного лабиринта (elevated plus-maze test – EPM) и тест социального взаимодействия (social interaction test – SIT), тест принудительного плавания (the forced swim test – FST) и тест горячей пластиной (hot plate test – HPT). В конце эксперимента измеряли прибавку в весе и жировые отложения (общие и забрюшинные). Определяли концентрацию липидов в сыворотке крови. Сывороточные концентрации фактора некроза опухоли альфа (TNF-альфа) и щёлочной фосфатазы (ALP) использовали для оценки воспаления и метаболизма костей, соответственно. Минеральную плотность бедренной кости (МПК) измеряли с помощью двухэнергетической рентгеновской абсорбциометрии (Dual Energy X-Ray Absorptiometry, DEXA).

Результаты: Крысы с OVX не показали значительных поведенческих изменений в тестах «открытое поле» и «поднятый перекрестный лабиринт», но у них уменьшилось время взаимодействия в тесте социального взаимодействия и увеличилось время иммобилизации в тесте принудительного плавания, что является индикатором беспокойства и депрессия. Крысы с OVX имели значительно более низкий порог болевой чувствительности. У них была более высокая прибавка в весе, увеличение общих и забрюшинных жировых отложений, а также соотношения общего жира / массы тела и забрюшинного жира / массы тела. Холестерин в крови, ЩФ и ФНО-альфа группы также были значительно выше. МПК бедренной кости крыс с OVX была не значительно увеличена.

Заключение: Дефицит эстрогена у крыс с OVX вызывал депрессию, беспокойство, гипералгезию, ожирение, дислипидемию и воспаление до того, как стало очевидным снижение минеральной плотности костей.

Ключевые слова

тревога, депрессия, ожирение, овариэктомия, крысы