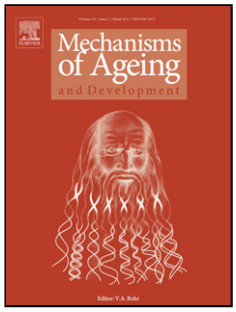

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Aging is associated with loss of beneficial effects of estrogen on leptin responsiveness in mice fed high fat diet: Role of estrogen receptor α and cytokines

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highlights

- High Fat Diet led to obesity in young ovariectomized compared to aged and young sham animals.
- Estrogen led to weight loss in young ovariectomized compared to aged animals.
- Estrogen increased leptin sensitivity in young animals, but not in aged.
- Estrogen modified the effects of HFD on brain levels of TNF-α and IL-10 in young ovariectomized and aged animals.
- Estrogen prevented the reduction of ERα caused by ovariectomy in ARC of young ovariectomized animals, but not in aged ones.

Abstract

Aging causes changes in body composition and energy balance. Estrogen plays an important role in body's metabolism. The aim of this study was to determine whether estrogen has beneficial effects on leptin responsiveness in aged mice. Young 4 months and aged 19-21 female mice fed High Fat Diet (HFD) or Standard Diet (SD) for 12 weeks and following received estrogen for 4 weeks. Responsiveness to leptin was compared by measuring energy balance parameters. Results showed that HFD caused weight gain compared to SD in young, but had no effect on aged animals. Estrogen reduced body weight, energy intake and visceral fat in young, while none of these parameters was affected in aged animals. Although there was leptin sensitivity in aged compared to ovariectomized animals, estrogen only improved the sensitivity of young to leptin. Estrogen prevented increase in TNF-α and a decrease in IL-10 in HFD young and aged animals. Response to estrogen depended on

age, and estrogen increased leptin sensitivity only in young animals. Determining the exact mechanism of this action is suggested in future studies.

Keywords: Aging, Estrogen, Leptin sensitivity, Cytokines, ERα**.**

1. Introduction

Aging, an unavoidable biological mechanism, is characterized by a decline in innate physiological functions that lead to reduced metabolic profile, changes in fat distribution, obesity, and insulin resistance (Tollefsbol and Cohen, 1986), all of which are associated with age-related diseases (Kotz et al., 1999). Leptin, secreted from white adipose tissue, is one of the most important energy balance regulating hormones (Park and Ahima, 2015), which results in reduced food intake and increased energy expenditure (Zhang and Scarpace, 2006). The effects of leptin on the brain and peripheral organs have been reported to decrease with aging. The redistribution of adipose tissue that occurs in old age may play a role in increasing the plasma level of leptin (Carter et al., 2013) and it seems that this hormone is one of the main causes of metabolic abnormalities in old age (Rosen and Bouxsein, 2006).

Also, aging creates changes in body composition, such as a change in fat mass, which involves a decrease in the size of fat cells in aged and middle-aged compared to young people (Fernández-Agulló et al., 2001). Studies show that leptin secretion profile is different in both young and aged people. In young state, leptin is predominantly secreted by subcutaneous fat, while in old age, leptin is secreted by visceral fat (Carter et al., 2013). It has been reported that despite an increase in the plasma level of leptin in old people, there is a disorder and weakness in the performance of leptin in old age that shows itself as a reduced sensitivity compared to young state (Gabriely et al., 2002).

Menopause or aging of the female reproductive system is a natural and physiological process and is referred to as natural aging (Troen, 2003). Estrogen acts on the CNS to modulate energy balance. Estrogen receptors (ERs) are widely expressed in the mice brain, particularly in hypothalamic sites, such as the arcuate nucleus (ARC), and paraventricular nucleus (PVH) (López and Tena-Sempere, 2017), all of which have key roles in the regulation of energy balance (Cornejo et al., 2016). Regarding the effect of estrogen on Agouti-related protein (AgRP)/Neuropeptide Y (NPY) neurons in the ARC, the available evidence is somewhat discrepant and conflictive (López and Tena-Sempere, 2017). Results of studied show that NPY levels are increased in ovariectomized (OVX) rat (animal model of menopause); and estrogen replacement correct it (López and Tena-Sempere, 2017). With the onset of menopause, along with the changes that occur with aging, estrogen production and its receptors undergo some changes, which can be associated with the emergence of age-related diseases , such as leptin resistance (Litwak et al., 2014), Alzheimer and Parkinson disease (Foster, 2012). Ovariectomy (model of menopause in rodents) has been reported to cause the imbalance in ERα and ERβ and an increase in ERβ signaling in peripheral tissue such as adipocyte, which has been suggested as a factor in weight gain in women after menopause (Tomicek et al., 2011).

Estrogen is an essential hormone in reproduction and plays an important role in regulating food intake and energy balance. The lack of estrogen in menopause and ovariectomy in rodents lead to fat accumulation and increased food intake, and estrogen therapy can reverse these conditions (Asarian and Geary, 2002, Blaustein and Wade, 1976, Hong et al., 2009). Moreover, the lack of estrogen in women causes metabolic disorders, including obesity, dyslipidemia, and insulin resistance (You et al., 2004, Kaaja, 2008). Studies have shown that ovariectomy causes leptin insensitivity and estrogen therapy can reverse this condition (Clegg et al., 2006). Redistribution of fat that happens in old age

can increase inflammation and disrupt the functional settings of fat cells (Carter et al., 2013). Obesity is associated with an increase in the expression of pro-inflammatory markers and inflammatory signaling in the hypothalamus of rat (Wang et al., 2012). It has been shown that the incidence of inflammatory diseases in women is lower than in men, and the differences in inflammatory responses between men and women are probably due to anti-inflammatory effects of estrogen (Foster, 2012).

Considering the above statements and also since in our previous study (Litwak et al., 2014) we found that estrogen decreases resistance to leptin in OVX obese animals, in this study, we determined whether the effects of estrogen on brain responsiveness to Leptin are age-dependent. Therefore, in order to test this hypothesis, the objectives of this study were: 1) to determine the effect of estrogen in increasing the sensitivity to leptin in young and aged mice, and 2) to determine the mechanism of estrogen effects by measuring inflammatory and anti-inflammatory cytokines in the brain, and also to investigate the changes in the expression of ERα as influencing factors in response to estrogen at different age.

2. Materials and Methods

2.1. Animals

Young, 4 months and aged 19-21 months (Matzel et al., 2008) female C57BL/6J mice were obtained from Pasteur Institute of Iran North Research Center. Animals were maintained at 22-23 °C on a 12:12-h light-dark cycle with free access to water and food ad libitum at the Animal Care Center of Kerman University of Medical Sciences. Control groups consumed Standard Diet (SD) (Pars Animal Feed, Iran) in which 5.7%, 72.2%, and 22.1% of calories are from fat, carbohydrates, and protein, respectively, i.e. a total caloric value of ~3.1 kcal/g. High Fat Diet (HFD) groups consumed HFD (Royan institute, Iran) in which 58.8%, 27.5%, and 14.7% of calories are from fat, carbohydrates, and proteins, respectively, a total caloric value of ~5.9 kcal/g. All experiments were performed in accordance with the ethical guidelines of the Kerman University of Medical Sciences Animal Ethics Committee (Permission No: 95/264 KA).

2.2. Bilateral ovariectomy procedures

Following anesthesia by Ketamine/Xylazine (80/10mg/kg intraperitonealy (i.p.)) a small incision was made in the abdomen lengthwise. Skin, fascia, and abdominal muscles were opened. Ovaries were appeared and removed. Then, 1-2 ml saline solution was poured in the abdomen and skins and muscles were stitched. In the sham surgery, similar incision was performed, but ovaries were not removed. Experiments started 2 weeks after ovariectomy (Azizian et al., 2018a).

2.3. Drugs

Ketamine and Xylazine were purchased from Alfasan Inc., Utrecht, Netherlands. 17-β estradiol (E2) and saline were obtained from Aburaihan Pharmaceutical (Tehran, Iran). Leptin and sesame oil were purchased from Sigma (St. Louis, MO, USA). E2 and leptin were dissolved in sesame oil and saline, respectively.

2.4. E2 treatment

Mice that were fed HFD for 12 weeks, received subcutaneous injection of E2 (2µg/mice in sesame oil; 100µl) (Litwak et al., 2014) or oil (100µl) as a control for 4 weeks. This was repeated every 4 days to mimic the estrus cycle (Litwak et al., 2014).

2.5. Locomotor activity measurement

Because it is possible that weight loss by E2, in addition to appetite loss, results from increased locomotor activity and increased energy expenditure, so to answer the hypothesis before and after E2 treatment open field test was measured. The mice were brought to the testing environment after 1 h acclimation period. Motor activity was measured using the open field test. All experiments were performed between 8:00 A.M. and 12:00 A.M. Distance traveled (horizontal activity) of mice was measured during a 30 min period in open field apparatus. The data was calculated during the last 20 min of the test, since exploratory and anxiety behavior is more than activity in the first few minutes of test (Musatov et al., 2007).

2.6. Leptin sensitivity test

To examine leptin sensitivity test, First, animals were anesthetized by mixture of Ketamine/Xylazine and placed in stereotaxic apparatus (USA, Illinois, Stoelting Co). Sterile cannula was inserted into the lateral ventricle (-0.5 mm posterior, 1 mm lateral to bregma, and 1.5 mm below the surface of the skull, in accordance with Franklin and Paxinos [Paxinos and Franklin, 2001]), 1 week is enough to recover from surgery, then recombinant murine leptin (1µL, 0.2 µg/µL: Sigma) (Litwak et al., 2014) or leptin's vehicle (saline) were injected into the lateral ventricle unilaterally. Finally, body weight and food intake were measured throughout the 48 h period following injection (Litwak et al., 2014).

2.7. Body fat determination

At the end of the study, visceral fat including (perirenal, mesenteric, retroperitoneal, perigonadal) and subcutaneous fat including (inguinal and interscapular) (Berry et al., 2013) was dissected and weighed.

2.8. Measurement of brain cytokines

At the end of E2 therapy period, animals were sacrificed, then the brains were quickly removed and immediately frozen in liquid nitrogen. The brains were weighed and homogenized in T-PER Tissue Protein Extraction Reagent with 0.5% Triton X-100, 150 mmol/L NaCl, 50 mmol/L Tris, and a protease inhibitor cocktail (Pierce). Following homogenization, the samples were centrifuged (4 °C and 4000g) for 15 min. The homogenate supernatant was collected. The protein content of the supernatant was estimated using a BCA Protein Assay Reagent Kit to ensure that an equal amount of protein from each sample was used for the assay (Taupin et al., 1993). Brain cytokines Tumor Necrosis Factor- alpha (TNF-α), Interleukin-6 (IL-6), and Interleukin-10 (IL-10) were also determined using commercially available ELISA kits (Hangzhou, Eastbiopharm, China) according to the manufacturer's instructions.

2.9. Determination of lipid profile

Fasting Plasma Glucose (FBG), Total Cholesterol (TC) and Triglyceride (TG) were measured after 12 h of fasting using commercial kits (Pars Azmun, Iran).

2.10. Immunohistochemical analysis

After 4 weeks' E2 treatment, animals were sacrificed, and brains were collected. For immunohistochemical staining, 2µm brain tissue sections were prepared from paraffin blocks and deparaffinized with xylene and hydrated by graded alcohols, washed with distilled water. Antigen retrieval was done by incubation with molar citrate buffer 1% (pH=6) in a microwave oven for 12 min and transferred to Phosphate Buffered Saline (PBS). To quench endogenous peroxidase activity, the sections were placed in 3% oxygen peroxide in methanol solution for 10 min. Then, sections were incubated with primary antibody (ERα (C-311): sc-787, Santa Cruz, USA, 1:500), for 60 min followed by incubation with secondary antibody and washed by PBS. Before being counterstained with

Hematoxylin, slides were incubated with DAB chromogen. Finally, slides were dehydrated in 70%, 90% and 100% ethanol, cleared with xylene and mounted.

2.11. Experiment 1

The schematic representation of the experimental protocol is illustrated in Fig. 1. To assess the effects of age on body weight, energy intake, and body composition, SD or HFD was given to animals for 12 weeks. In this study, body weight and energy intake (food intake) was measured weekly and daily, respectively. The young ovarian-intact (sham), young ovariectomized (OVX) and aged mice were randomly divided into two groups of HFD and SD. The classification of animals was as follows (n=12): sham mice that were fed SD (Sham+SD), OVX mice that were fed SD (OVX+SD), aged mice that were fed SD (Aged+SD), sham mice that were fed HFD (Sham+HFD), OVX mice that were fed HFD (OVX+HFD), and aged mice that were fed HFD (Aged+HFD).

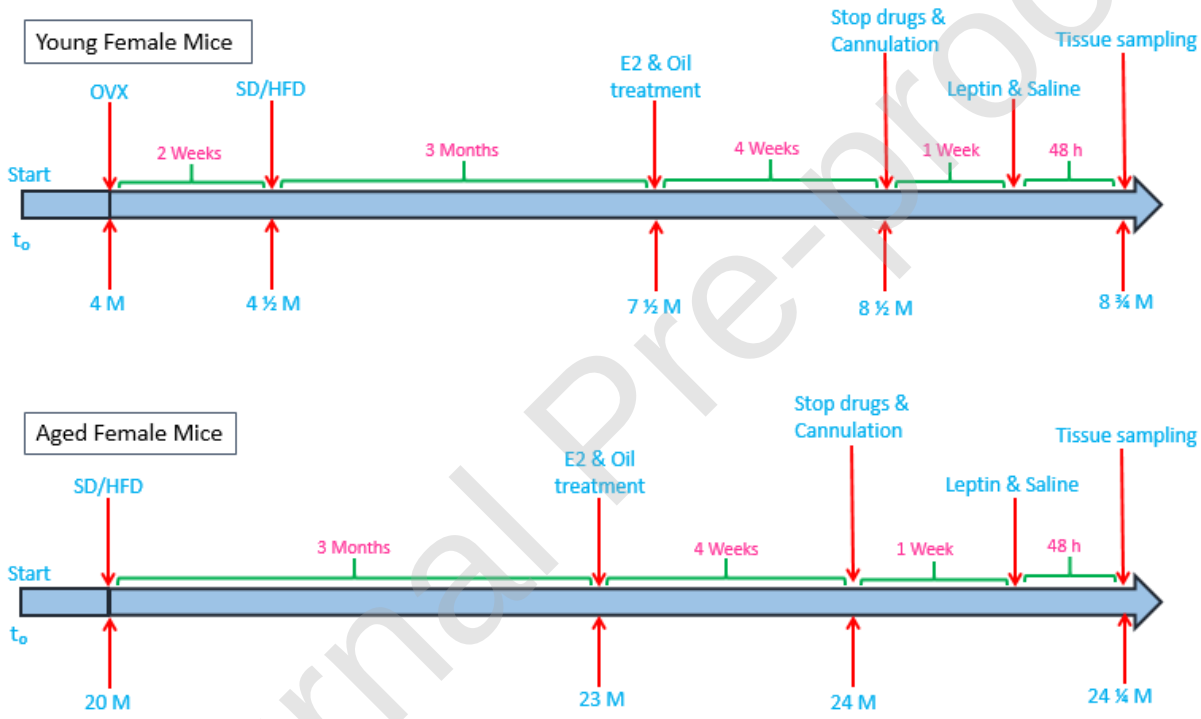


Figure 1. Schematic representation of the experimental protocol. E2: 17β Estradiol, HFD: High fat diet, M: Months, OVX: Ovariectomy, SD: Standard diet**.**

2.12. Experiment 2

At the end of 12 weeks, to assess the effects of exogenous E2 on body weight, energy intake, and body composition in young and aged animals that were fed HFD, the animals were randomly assigned into six groups of 12 animals each. 1) E2-treated sham mice that were fed HFD (Sham+HFD+E2), 2) Oil-treated (E2 solvent) sham mice that were fed HFD (Sham+HFD+OIL), 3) E2-treated OVX mice that were fed HFD (OVX+HFD+E2), 4) Oil-treated OVX mice that were fed HFD (OVX+HFD+OIL), 5) E2-treated aged mice that were fed HFD (Aged+HFD+E2), and 6) Oil-treated aged mice that were fed HFD (Aged+HFD+OIL).

2.13. Experiment 3

To assess whether E2 increases central sensitivity to leptin, or whether E2-induced weight loss is a factor in this increase, a Pair Body Weight (PBW) group was also designed, in addition to young E2-treated animals. The PBW group included the Sham-PBW and OVX-PBW, which received the oil-treatment but their diet was adjusted so that, their weight at the end of the study was similar to that of E2-treated animals, and finally, their body weight and energy intake were measured 48 h after the injection of leptin.

2.14. Experiment 4

At the end of the study, in order to assess the level of response to leptin in the presence of E2, leptin was administered to the animals (n=8) of the second experiment and 48 h later, their body weight and energy intake were measured.

2.15. Statistics

Data were analyzed by two-way ANOVA repeated measurement for body weight change and energy intake over time followed by Bonferroni post hoc test. One-way ANOVA was used to analyze visceral, subcutaneous fat mass, ratio visceral/subcutaneous, and changes of body weight in fig. 4. Other parameters were analyzed by two-way ANOVA, followed by Bonferroni multiple comparisons using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Results are presented as mean ± S.E.M and $P < 0.05$ was considered statistically significant.

3. Results

3.1. HFD resulted in weight gain in young but not in aged animals

The comparison of animals' body weight changes in different groups fed SD or HFD are shown in supplemental data. Ovariectomy leads to weight gain, thus in this study, the consumption of both SD and HFD caused more weight gain in young OVX compared to young sham animals. The weight of Aged+HFD animals at the end of the study was more than the Sham+HFD animals, which was due to their higher weight at the beginning of the study. The energy intake (kcal/day) in young Sham+HFD and young OVX+HFD was significantly higher than the Aged+HFD. However, energy intake (kcal/day) in young sham, young OVX and Aged that were feed SD did not differ significantly (supplemental data).

Changes in the level of visceral fat in different groups are shown in supplemental data. Ovariectomy in the OVX+SD resulted in a greater increase in visceral fat compared to the Sham+SD and Aged+SD groups. HFD significantly increased visceral fat in young OVX compared to young sham animals, as well as aged animals. The consumption of HFD, in comparison with the SD in young OVX animals caused a 3.5-fold increase in visceral fat.

Similar to visceral fat, the subcutaneous fat in OVX+HFD group was higher than Sham+HFD and Aged+HFD. Subcutaneous fat in OVX+SD was higher than Aged+SD and Sham+SD. On the other hand, the levels of subcutaneous fat in aged animals with different diets did not differ significantly (supplemental data).

Further analysis of fat content showed that the ratio of visceral fat to subcutaneous fat in the OVX+HFD and Aged+HFD groups was higher than the Sham+HFD (supplemental data). Also, the ratio of visceral to subcutaneous in Aged+SD was higher than both young groups fed SD. Although this ratio was significantly different in the OVX+HFD group compared to the OVX+SD group, this difference did

not exist in aged animals that were fed both SD and HFD. The increase in this ratio in OVX+HFD group seems to be caused by the accumulation of visceral fat.

3.2. The effects of E2 on the uterus weight and distance traveled of young and aged animals

The distance traveled and uterus weight of young and aged animals after 4 weeks of treatment with E2 is shown in Table 1. E2 increased distance traveled compared to oil in the OVX animals ($P<0.05$). However, there was no significant difference in distance traveled between E2 and oil in the Aged and sham groups after E2 treatment. In OVX and aged, the uterus weight decreased compared to sham animals($P<0.001$). Also, treatment with E2 increased the uterus weight in young sham and young OVX animals ($P<0.001$).

3.3. The effects of leptin on body weight and energy intake in young and aged animals with different diets

Leptin reduced the body weight of young sham animals that were fed both SD and HFD compared to saline ($P<0.001$, Fig. 2A). Also, leptin in the OVX+SD group caused a significant decrease in body weight compared to the saline group ($P<0.001$, Fig. 2B). This is while, leptin was not able to reduce body weight in the OVX+HFD group compared to the saline group, which meant that a resistance to leptin has been created in animals fed HFD. Additionally, leptin injection led to weight loss in the Aged+SD and Aged+HFD groups ($P<0.05$, Fig. 2C), which meant that HFD in aged animals did not lead to resistance to leptin. As above, leptin led to decrease in energy intake (kcal/day) in Sham+SD and Sham+HFD groups ($P<0.05$, Fig. 2D). Also, leptin reduced energy intake (kcal/day) in OVX+SD compared to the saline group ($P<0.05$, Fig. 2E). Finally, leptin decreased energy intake (kcal/day) in both Aged+SD and Aged+HFD groups compared to saline ($P<0.001$, Fig. 2F).

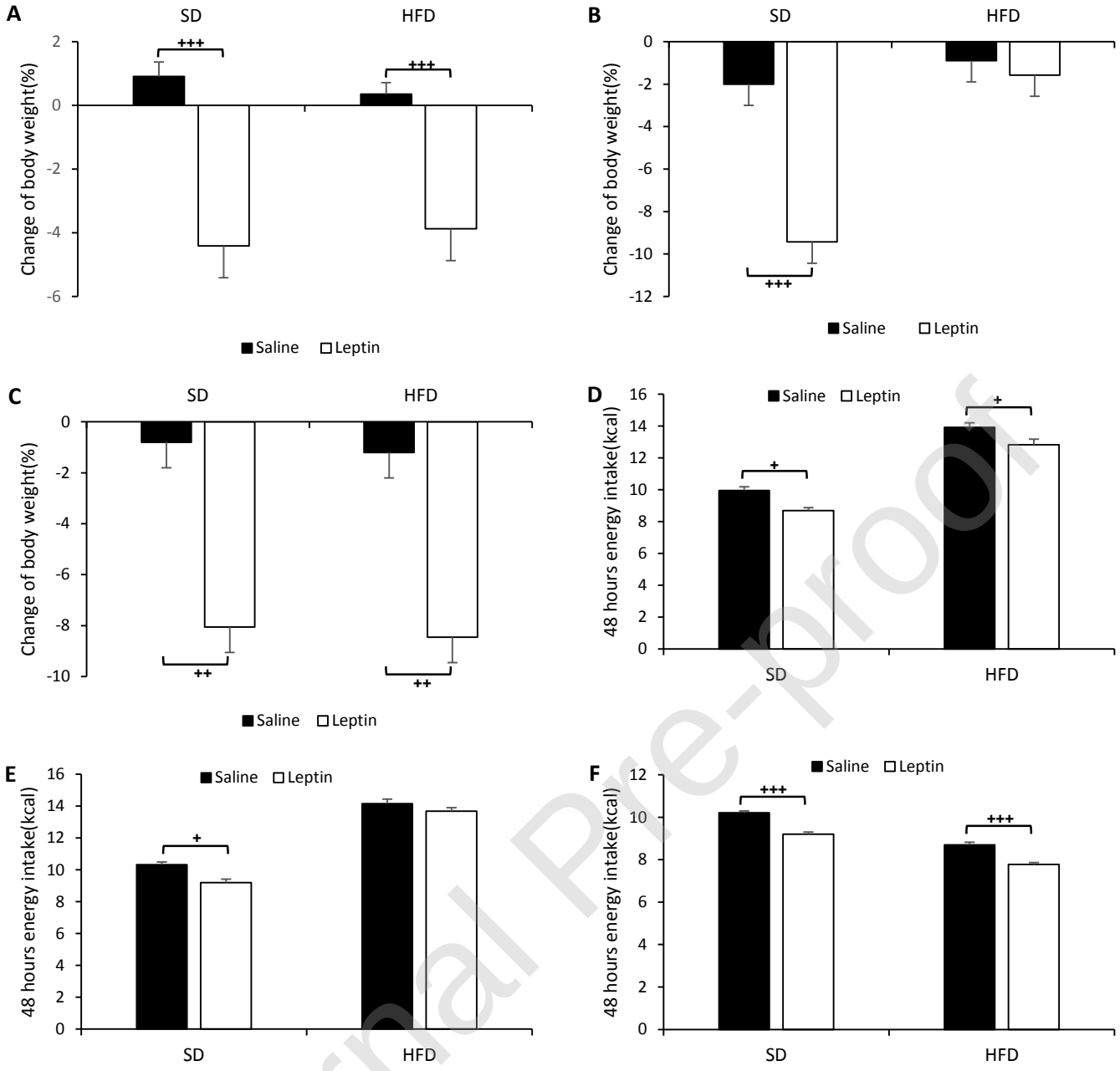


Figure 2. The effect of central leptin (0.2µg/µl) on body weight and energy intake during 48 hours after a single injection of leptin compared with saline. Change of body weight (%) in (A) young sham, (B) OVX, and (C) Aged animals were fed by HFD or SD. Energy intake 48 h after i.c.v saline or leptin (0.2µg/µl) injection in (D) young sham, (E) OVX, and (F) Aged animals fed by SD or HFD. Results are expressed as mean ± SEM., n=8. $^{+}P<0.05$, $^{++}P<0.01$ and $^{+++}P<0.001$. HFD: High Fat Diet, OVX: Ovariectomy, SD: Standard Diet, Sham: Ovarian intact.

3.4. The effects of E2 therapy on body weight, energy intake, and fat mass in young and aged female mice fed with HFD

The changes in the body weight of animals in various groups fed with HFD during 4 weeks of E2 and oil injections are shown in (Fig. 3A and 3B). E2 treatment of young sham and young OVX animals that were fed HFD reduced the body weight compared to young sham and young OVX groups fed with HFD treated by oil ($P<0.001$). Weight loss in the OVX group was observed from the first week of treatment. On the other hand, as in the E2-treated group, OVX+PBW group had lower body weight compared to the OVX+oil group ($P<0.001$). Aged animal's treatment with E2 did not change the body weight compared to oil.

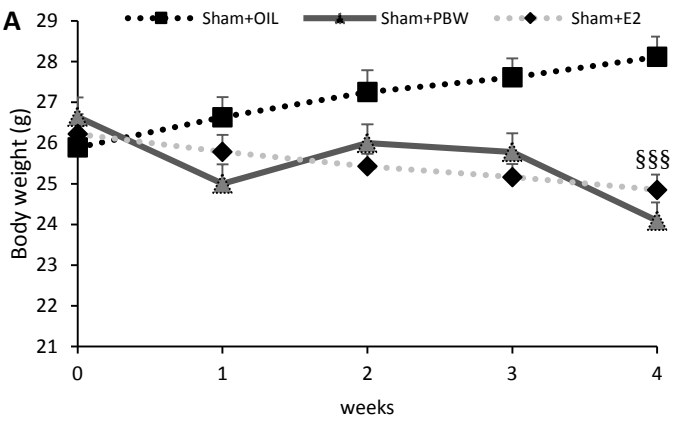
(Figure 3C and 3D) shows the daily energy intake in different groups during the 4 weeks of treatment with E2 and oil. Energy intake (kcal/day) in the Sham+E2 group was lower than the Sham+OIL group ($P<0.001$). Also, E2 in OVX fed with HFD group reduced the energy intake (kcal/day) in comparison with the oil ($P<0.01$), although aged animals did not differ from each other in this index, which meant E2 did not have any effect on energy intake in the aged group.

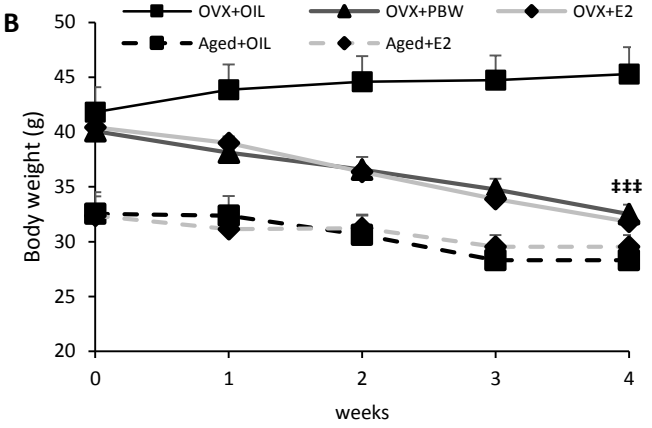
E2 reduced the level of visceral fat compared to oil in sham animals ($P<0.01$, Fig. 3E). Level of visceral fat was higher in oil group than the E2 and PBW groups in OVX animals ($P<0.001$, Fig. 3E). The level of visceral fat in the Aged+OIL group was 1.3 ± 0.23g, which was not changed by the E2 treatment. This is consistent with the lack of E2 effect on the weight of treated aged animals.

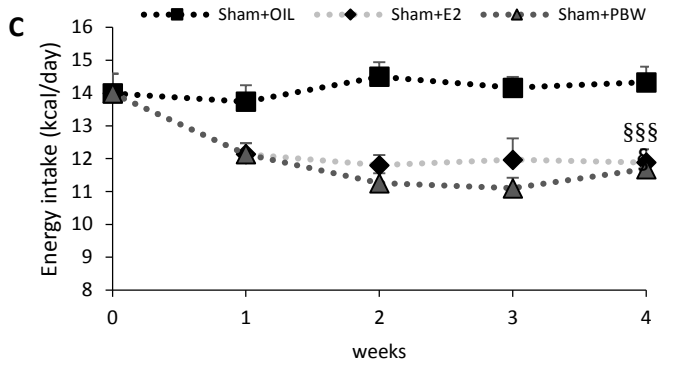
As with visceral fat, E2 decreased the level of subcutaneous fat compared to oil group in sham animals ($P<0.01$, Fig. 3F). On the other hand, although the level of subcutaneous fat increased in the OVX+OIL group, E2 consumption reduced the level of subcutaneous fat ($P<0.01$, Fig. 3F). E2 seems to have an impact on weight loss by reducing both types of fat. It should be noted that, similar to visceral fat, the level of subcutaneous fat was not affected by E2 in aged animals (Fig. 3F).

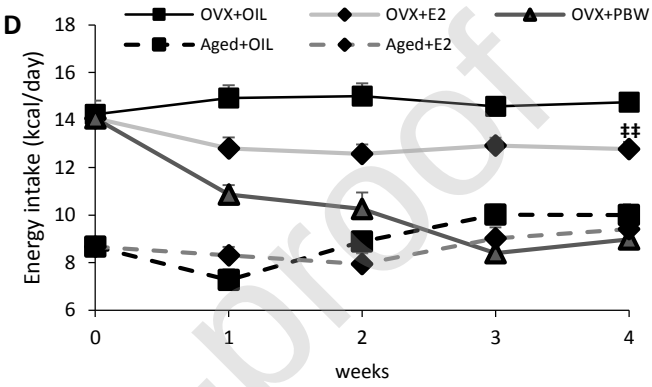
Further analysis of fat in Fig. 3G shows the changes in the ratio of visceral to subcutaneous fat in the various groups. As seen, this ratio was decreased in the OVX+E2 group, meaning that, E2 therapy led to a greater reduction in the level of visceral fat (70%) compared to subcutaneous fat (50%).

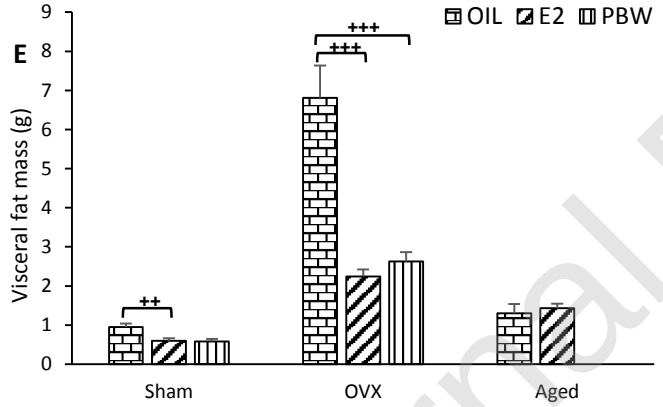
The percentage of body weight changes in young and aged animals in the presence of E2 is shown in Fig. 3H. The percentage of weight loss in the OVX+E2 was higher than the Sham+E2 ($P<0.01$). Similarly, comparing the percentage of body weight changes in OVX and Aged groups treated with E2 showed a significant difference in the function of E2 ($P<0.001$).

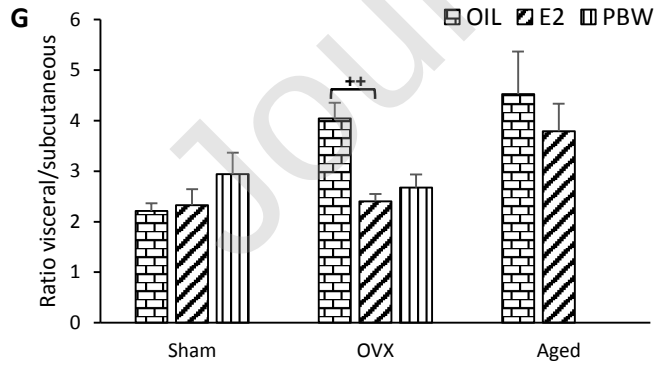












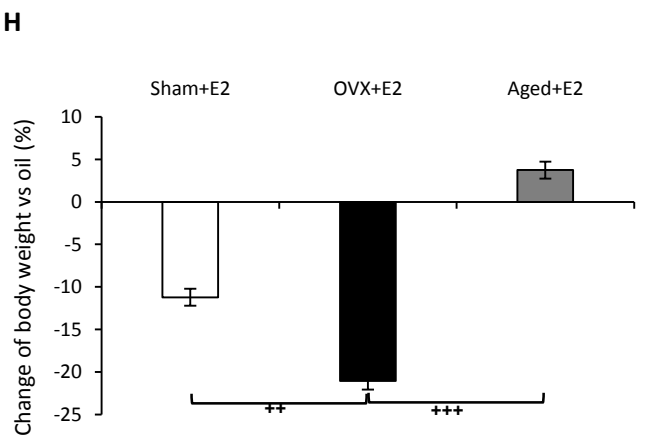


Figure 3. The effect of chronic administration of E2 or oil on (A and B) body weight, (C and D) energy intake, (E) visceral fat, (F) subcutaneous fat, (G) ratio visceral to subcutaneous fat, and (H) body weight change (%) in young (Sham and OVX) and Aged HFD fed mice. Results are expressed as mean ± SEM., n=12 mice in A, B, C, D and H, and 8 mice in E, F. §§§P<0.001 vs. Sham+OIL, ‡‡P<0.01, ‡‡‡P<0.001 vs. OVX+OIL. ++P<0.01 and +++P<0.001. E2: 17-β estradiol, OVX: Ovariectomy, PBW: Pair Body Weight, Sham: Ovarian intact.

3.5. The effects of E2 on the level of leptin sensitivity in young and aged animals

To examine the hypothesis that E2 can return leptin sensitivity in obese mice, the percentage of body weight changes in different groups was measured 48 h after the injection of leptin and saline, as shown in Fig. 4. Leptin injection led to weight loss in the Sham+E2, Sham+OIL and Sham+PBW groups compared to the saline group (P<0.001, Fig. 4A). According to the results of this study, there was a significant difference in response to leptin between the Sham+E2, and Sham+OIL groups (P<0.05, Fig. 4A), but no significant difference was found between the Sham+E2 and Sham+PBW groups. Similarly, leptin injection resulted in weight loss only in the OVX+E2 and OVX+PBW groups (P<0.001, Fig. 4B). There was also a significant difference in response to leptin between the OVX+E2 and OVX+OIL groups, and also, OVX+E2 and OVX+PBW (0.001, Fig. 4B), meaning that E2 returned the sensitivity to leptin. On the other hand, the results for aged animals were similar to those of OVX+E2 animals, so that, leptin injection led to weight loss in both Aged+OIL and Aged+E2 (P<0.001, Fig. 4C). There was no difference between the E2 and oil groups in terms of response to leptin, which meant that leptin sensitivity did not disappear in aged animals, and this effect of leptin was not associated with E2. Leptin injection in the OVX+E2 and OVX+PBW groups caused a reduction in energy intake compared to saline (respectively P<001 and P<0.05, Fig. 4E), and such as body weight, there was significant difference in response to leptin between OVX+E2 and OVX+OIL groups and also, OVX+E2 and OVX+PBW (P<0.001, Fig. 4E), and leptin did not make any difference in the level of energy intake between the E2 and the oil groups in aged animals.

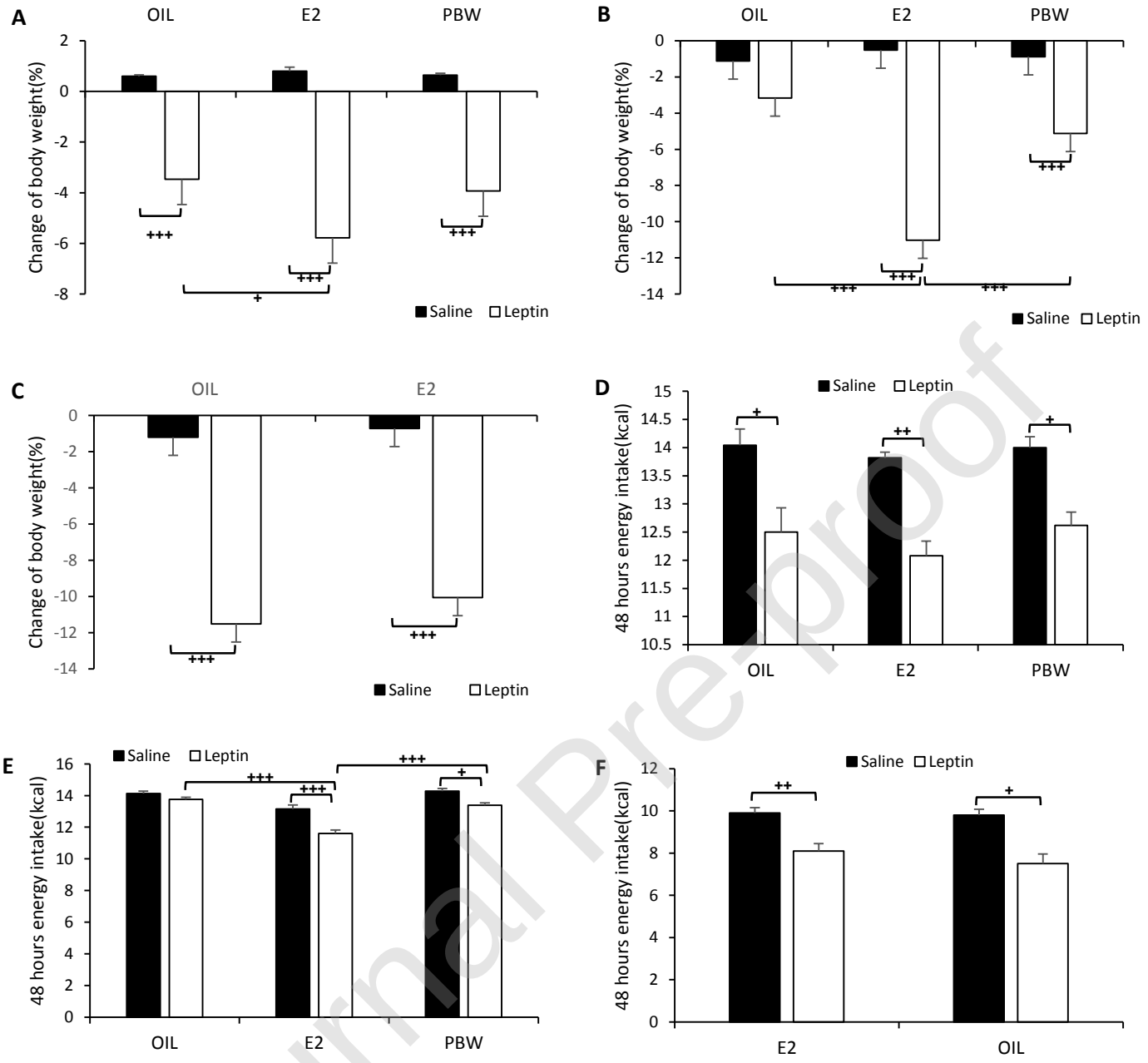


Figure 4. The effect of central leptin (0.2µg/µl) on body weight and energy intake in HFD fed mice treated by E2 and oil during 48 hours after a single injection of leptin compared with saline. Body weight change (%) in **A:** young sham **B:** OVX **C:** Aged animals were fed by HFD. Energy intake 48 h after i.c.v saline or leptin (0.2µg/µl) in **D:** young sham **E:** OVX and **F:** Aged animals fed by HFD. Results are expressed as mean ± SEM., n= 8. $^{+}P<0.05$, $^{++}P<0.01$, and $^{+++}P<0.001$. E2: 17-β estradiol, OVX: Ovariectomy, PBW: Pair Body Weight, Sham: Ovarian intact.

3.6. E2 modified levels of brain cytokines in young and aged animals

The changes in cytokines in various groups of the study are shown in Fig. 5. In young sham animals, E2 therapy was unable to change the cytokines compared to the oil and HFD groups. In the young OVX animals, E2 therapy decreased the levels of TNF-α ($P<0.05$, Fig. 5A) and IL-6 ($P<0.001$, Fig. 5B) and increased the level of IL-10 ($P<0.05$, Fig. 5C) compared to the oil group. Similar to the above results, E2 also decreased the levels of IL-6 ($P<0.01$, Fig. 5B) and TNF-α ($P<0.001$, Fig. 5A) and increased the level of IL-10 ($P<0.05$, Fig. 5C) compared to the OVX+HFD group. Treatment with E2 in aged animals compared to oil reduced the level of TNF-α ($P<0.05$) and increased the level of IL-10 ($P<0.05$) (Fig. 5A and 5C), but did not have any impact on the reduction of IL-6 level.

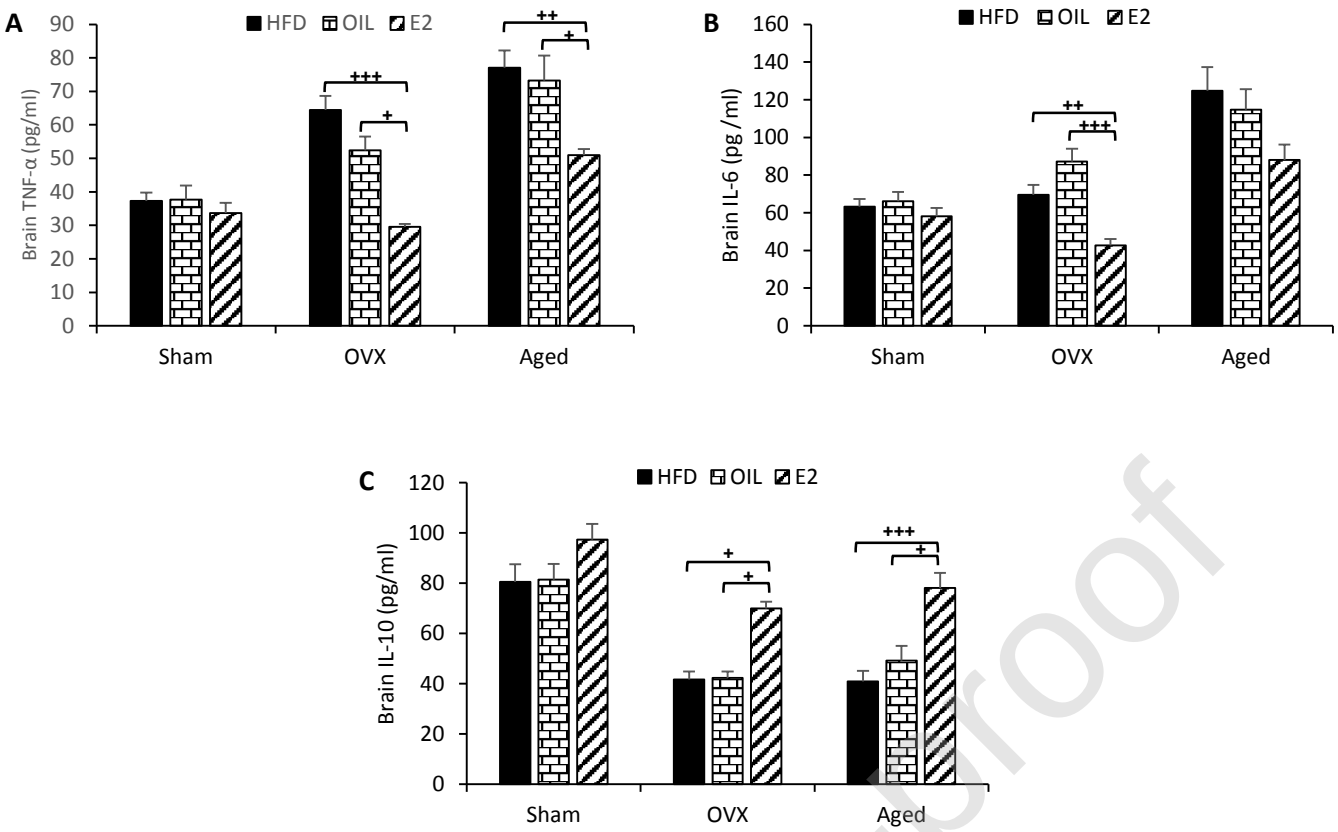


Figure 5. The effect of chronic E2 or oil treatment on levels of inflammatory and anti-inflammatory markers in the whole brain of young (Sham and OVX) and aged mice were fed by HFD. **A:** levels of TNF-α **B:** IL-6 **C:** IL-10. Results are expressed as mean ± SEM., n= 6. $^{+}P<0.05$, $^{++}P<0.01$, and $^{+++}P<0.001$. E2: 17-β estradiol. HFD: High Fat Diet, OVX: Ovariectomy, PBW: Pair Body Weight, Sham: Ovarian intact.

3.7. The effects of E2 on lipid profile and FBG in young and aged animals

The lipid profile and FBG in young and aged animals fed with different diets are shown in Table 2. The consumption of HFD compared to SD in young sham animals resulted in elevated levels of TC, TG ($P<0.001$) and FBG ($P<0.05$). Treatment with E2 only reduced the TC ($P<0.001$, Table 2) compared to oil. Consumption of HFD in OVX mice led to increasing the level of TC, TG and FBG compared to the SD ($P<0.001$, Table 2). Treatment with E2 in OVX animals reduced the level of TC and FBG compared to the oil ($P<0.001$, Table 2). On the other hand, in aged animals, HFD resulted in increased TC and FBG level ($P<0.001$, Table 2), while E2 therapy did not have any impact on these variables.

3.8. Changes of ERα expression at ARC in presence of E2 in young and aged animals

In the last part of the study, the changes in the ERα were examined to determine the possible mechanism of E2 effect. Results showed that the ovariectomy decreased expression of ERα and E2 treatment increased the ERα in the ARC (Fig. 6). In aged animals, the expression of ERα decreased, and unlike OVX+OIL, treatment with E2 was not able to increase the expression of it (Fig. 6).

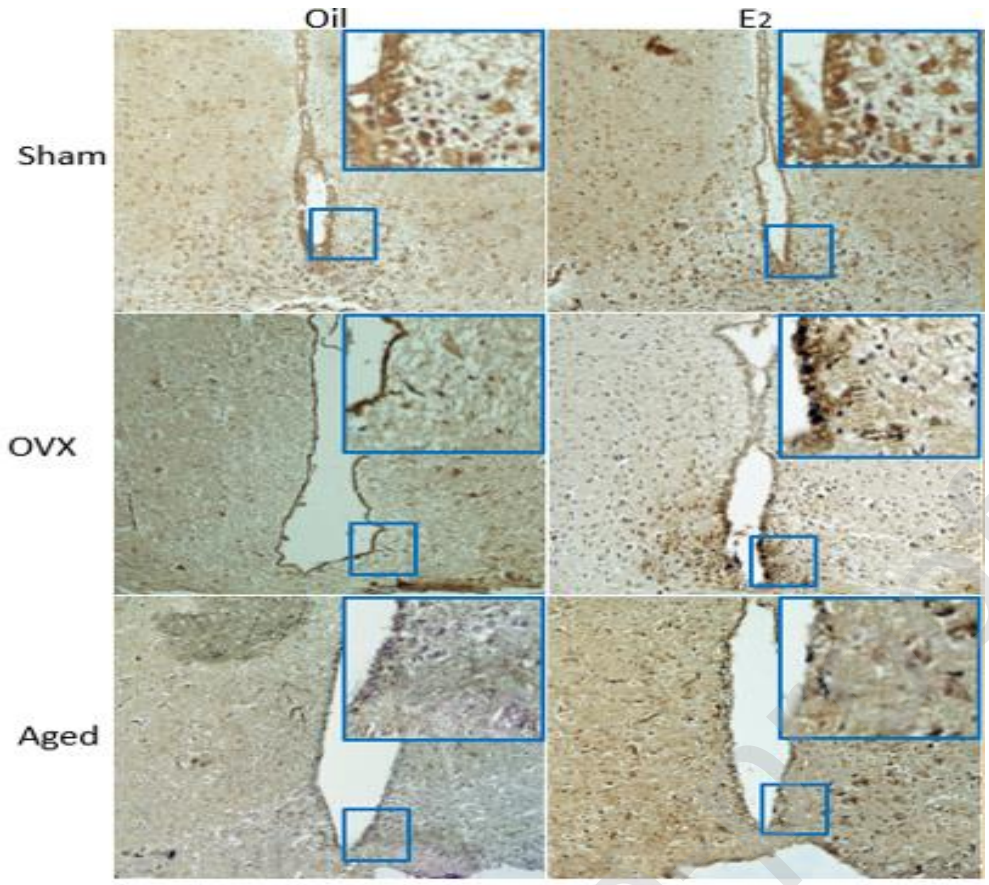


Figure 6. Microscopic IHC sections of hypothalamus (ERα staining) of various groups of animals fed with HFD at the end of study. E2: 17-β estradiol, OVX: Ovariectomy, Sham: Ovarian intact.

4. Discussion

The prevalence of obesity in aged and postmenopausal women is higher than in pre-menopause women, which is probably due to the lack of E2 or disturbances in E2 signaling. The aim of this study was to examine the effects of E2 on the metabolic and anthropometric outcomes caused by HFD in young and aged female mice. The main findings of the present study include: 1) HFD led to body weight gain, increased energy intake, and increased visceral and subcutaneous fat in young OVX animals compared to aged animals and young sham animals. 2) Chronic administration of E2 led to weight loss, reduced energy intake, visceral, and subcutaneous fat in young OVX compared to aged animals. 3) E2 in young animals increased the effects of leptin on weight loss and energy intake, although it did not have any impact on leptin sensitivity in aged animals. 4) HFD increased the levels of TNF-α and IL-6 and decreased the level of IL-10. E2 prevented these effects in young OVX animals, while it failed to resolve the effects of HFD on the level of IL-6 in aged animals. 5) E2 prevented the reduction of ERα caused by ovariectomy in ARC of young OVX animals, but not in old ones.

HFD consumption not only induces obesity in animals but also leads to obesity in humans (Rothwell and Stock, 1984). A positive correlation between fat percentage in food, weight gain, and obesity has been reported both in the rat (Boozer et al., 1995) and in mice (Bourgeois et al., 1983). In agreement with our study, it has been reported that HFD consuming mice are more susceptible to obesity, and the energy in HFD turns into fat and causes obesity (Ludgero-Correia et al., 2012). It has also been reported that the ovariectomy exacerbates weight gain, for example, weight gain in OVX animals

compared to sham animals fed with HFD and SD (Litwak et al., 2014). The possible mechanism(s) by which, ovariectomy causes weight gain include: increased susceptibility for fat accumulation (Hong et al., 2009), changes in the expression of PPAR and Fox genes (Rogers et al., 2010), and a dramatic reduction in basal metabolism (Musatov et al., 2007).

The weight loss of animals following E2 administration that occurred in the present study could be due to decreased food intake (Rogers et al., 2009), increased anorexigenic and decreased orexigenic signaling (Brown and Clegg, 2010), and decreased NPY activity (Argente-Arizón et al., 2017). There are probably other mechanisms for weight loss caused by E2 in addition to those mentioned above, as it has been reported that ovariectomy leads to weight gain even after pair-feeding (Dafopoulos et al., 2010). Moreover, moderated fat metabolism (Roy and Wade, 1977), and increased energy expenditure have also been reported in various studies. In another part of our study, according to locomotor activity results of open field test, it was found that E2 may also decrease weight by increasing locomotor activity. Because E2 increased the distance traveled in the open field. The results are consistent with our study that E2 increases locomotor activity (Musatov et al., 2007, Mauvais-Jarvis et al., 2013) . Since E2 increases the production of α.MSH (Mauvais-Jarvis et al., 2013, Litwak et al., 2014), perhaps increased energy expenditure is also stimulated through this peptide. Because the results of the recent studies indicate that the melanocortin system induces sympathetic activity, it increases metabolism and reduces food intake (Merchenthaler et al., 2004).

Menopause in women and a model of menopause in rodents directly lead to fat accumulation (Litwak et al., 2014). Following ovariectomy, there will be a greater increase in visceral fat accumulation relative to subcutaneous fat (Rogers et al., 2009). Yonezawa et al (Yonezawa et al., 2012) reported that ovariectomy in mice fed with HFD leads to weight gain, followed by visceral and subcutaneous fat accumulation. The fat accumulation has also been seen in sham mice that were fed HFD (Yonezawa et al., 2012, Al-Qahtani et al., 2017), although their fat content was less than that of OVX mice (Litwak et al., 2014).

Chronic E2 administration prevented weight gain in OVX animals that were fed with HFD. Consistent with the current study, it has been reported that E2, in addition to having a direct effect on adipocytes (Tara et al., 2005), also reduces abdominal fat (Stubbins et al., 2012). E2 reduces fat mass may be by inhibiting the genes involved in lipogenesis and adipogenesis (Cooke and Naaz, 2004). Other possible mechanism(s) for such effects of E2 include, reducing the synthesis of fatty acids and TG (Tara et al., 2005), regulating ERs, and reduced inflammation (Brown and Clegg, 2010).

Unlike the young OVX, that HFDs increased their weight, there was no change in the weight of aged animals, although the energy intake by them was less than that of OVX animals. In line with the current study, it has been reported that, although obesity is one of the major problems of old age, its prevalence decreases with advanced old age (Kennedy et al., 2004), so that body weight usually increases until middle age (Lei et al., 2006), and then begins to decrease (Baumgartner et al., 1995). Additionally, it has been shown that total body fat or body fat percentage begins to decrease in men and women after the age of 70 years (Raguso et al., 2006, Visser et al., 2003). It has also been reported that body weight and muscle mass in c57 mice begin to decrease between the age of 18 and 24 months (Hamrick et al., 2006). Some studies have referred to reduced food intake, increased metabolism (Thompson and Morris, 1991), reduced muscle mass, and increased level of inflammatory cytokines (Hamrick et al., 2006). There is a report that indicates, unlike the present study, HFD consumption has been associated with weight gain in aged animals (Bailey-Downs et al., 2012), which is probably due to the difference in sex of animals compared to our study.

HFD or SD diets increased visceral and subcutaneous fat in young OVX animals, but this did not occur in aged animals. In addition, comparison of subcutaneous fat in aged animals indicated that the level of this fat was even lower than the young sham animals, and the ratio of visceral to subcutaneous fat in aged animals was higher than young animals. Low levels of leptin in aged animals have been reported to be due to the animals' low fat content (Perry III et al., 1997). Although the fat percentage has been reported to decrease in men and women after the age of 70 years (Raguso et al., 2006), this decrease in body fat occurs more in the subcutaneous region than in the visceral region (Hughes et al., 2004).The possible mechanism(s) for such changes in fat that are associated with aging include; reduced the adipocytes' size (Kirkland and Dobson, 1997), fat accumulation outside of the adipose tissue (redistribution) (Zamboni et al., 1997, Carter et al., 2013), and impaired function of adipose tissue (Cartwright et al., 2007).

In aged animals, unlike young OVX animals, E2 did not have a beneficial effect and did not make any changes in the weight, visceral fat and food intake, which could probably be due to changes in the level of ERα receptors in hypothalamus and other parts of the brain that occur with aging (Nelson et al., 1995, Thakur and Sharma, 2007, Adams et al., 2002, Bao et al., 2006). In another part of this study, it was shown that ovariectomy reduced ERα in ARC, while E2 therapy in these animals prevented the ERα reduction. However, in aged animals, E2 did not show such ability. The hypothalamus is a data center for regulating energy balance (Gali Ramamoorthy et al., 2015). E2 has been shown to alter the expression of ERα in the POMC neurons in the ARC (Wise et al., 1990). Also, changes in ERα expression following E2 therapy have also been reported in other organs (Azizian et al., 2018a). However, contrary to the present study, E2 therapy has been reported to reduce ERα, which is probably due to differences in E2 dosage and type of animal (Lauber et al., 1990).

E2 was found to induce its anti-inflammatory effects by reducing TNF-α and increasing IL-10 in the brain in young OVX as well as aged animals that were fed HFD. An increase in the expression of IL-6 and TNF-α in the hypothalamus after the consumption of HFD has been reported (Wang et al., 2012). Consistent with our results, E2 therapy has been reported to often result in an increase in IL-10 level (Azizian et al., 2018b). It has also been reported that E2 reduces the elevated production of TNF-α caused by aging (Sadagurski et al., 2017). The mechanism(s) by which, E2 improves the levels of inflammation markers include: increasing IkB and inhibiting NFkB signaling pathway (Yang et al., 2006). It seems that one of the mechanisms by which, E2 prevents weight gain and reduces food intake is to make changes in inflammation by altering the production of inflammatory cytokines or their signaling pathway in the hypothalamus. It should be noted that in our study ovariectomy reduced ERα and increased inflammation in the brain and E2 therapy prevented these effects. Therefore, it is likely that E2 and ERα play a protective and anti-inflammatory role in the brain. In vitro studies have reported that E2 activates ERα and reduces the pro-inflammatory cytokines (Vegeto et al., 2003). However, the mechanism of cytokines in regulating appetite appears to be different in young and aged animals. It has been reported that high level of cytokines in aged animals suppresses the appetite and catabolism of muscle proteins, which ultimately leads to weight loss and muscle weakness (Morley, 2001). because many of these cytokines belong to the same superfamily as leptin and are thought to produce their anorectic effects by stimulating the leptin receptor (Zhang et al., 1997).

Results of this study showed that HFD led to leptin resistance only in young OVX animals and because in our study, aged animals were not only obese but also, weight loss, increased the sensitivity to leptin. Ovariectomy reduced the sensitivity to leptin, while E2 therapy in young OVX animals led to restoration in leptin sensitivity in spite of consumption of HFD. In line with the current study, it has been reported that consuming HFD in OVX animals leads to leptin resistance, and E2 injection return leptin sensitivity in OVX, which perhaps due to the normalized expression of leptin receptor in the

brain (Matyšková et al., 2010). Another possibility is the prevention of weight gain by E2 that was not confirmed in our study, because in young animals with the same weight, although their weights were the same as the weights of animals in E2 group, their sensitivity to leptin was lower. This finding rejects the hypothesis that leptin sensitivity does not change in aged animals due to the lack of weight gain (Balaskó et al., 2014). Although this mechanism, which E2 was not effective in increasing leptin sensitivity in aging, could be one of the possible mechanisms of leptin effects in aged animals, as it has been reported that in the absence of obesity, leptin sensitivity in aged animals not only will not reduce but also will increase (Balaskó et al., 2014, Hosoi et al., 2005).

In another part of the study, it was shown that the levels of TC and TG increased in both young and aged animals fed HFD compared with SD, and E2 only reduced the TC level in young animals. In agreement with the present study, it has been reported that HFD and ovariectomy increase TC and TG levels (Hamza and Farag, 2011). Korou et al (Korou et al., 2013) showed that increases of FBG and TC in young mice after the consumption of HFD are less than that of aged mice. The cause of TC and glucose increase after menopause is a reduction in insulin sensitivity, which is associated with increased body fat mass and inflammatory markers (Mauvais-Jarvis et al., 2013). So perhaps this increase in insulin resistance and inflammatory markers is one of the reasons for weight gain after the consumption of HFD. The possible mechanism(s) through which E2 regulates lipid profiles include: reducing body weight and deposition of fat (Camara et al., 2014) and effects on genes involved in the metabolism of lipoproteins (Mauvais-Jarvis et al., 2013).

5. Conclusion

In summary, the results of this study showed that consumption of HFD in young OVX animals increased energy intake and body weight compared to aged animals. These problems were eliminated by E2 administration in young OVX animals, while E2 had no effect on aged animals. A possible mechanism for such effects of E2 is through the increase in ERα in the ARC because both ovariectomy and normal aging reduce the receptors in the ARC. E2 only in young animals was able to increase these receptors. On the other hand, HFD resulted in increased visceral and subcutaneous fat only in young animals. In aged animals, the ratio of visceral fat to subcutaneous fat increased due to reduced subcutaneous fat. Another possible mechanism for E2 effects in young animals is the reduction in inflammatory cytokines and increase in anti-inflammatory cytokines. Although in aged animals, E2 caused similar changes in cytokines, it did not affect the weight and energy intake. Leptin sensitivity was reduced in the OVX animals that fed HFD, and E2 only in these animals was able to increase the sensitivity to leptin and did not have any effect on aged animals. It is suggested that future studies should compare the effects of E2 in young and middle-aged animals, and investigate the effects of E2 on other aspects, such as insulin resistance in these two age groups. Also, the non-genomic pathway for E2 effects in young and aged animals along with the post-receptor mechanisms of ER also need to be studied. In addition, whether selective estrogen receptor modulators (SERMs) could serve as therapeutic targets for the treatment of menopausal weight gain.

Conflict of interest

The authors declare that there is no conflict of interests.

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Table 1:
The effects of E2 on the uterus weight and distance in young and aged animals.

Parameters	Sham		OVX		Aged	
	OIL	E2	OIL	E2	OIL	E2
UW (g)	0.077 ±0.004	0.14 ±0.01§§§	0.008± 0.001	0.065± 0.002‡‡‡	0.013± 0.003	0.017± 0.005
Distance traveled (cm)	13497±204	13781±98	11490±1017	13996±575‡	12076±500	11457±724

Results are expressed as mean ± SEM., n= 6 mice/group. ‡ P<0.05 and ‡‡‡ P< 0.001 vs. OVX+OIL. §§§ P<0.001 vs. Sham+OIL. E2:17-β estradiol, OVX: Ovariectomy, Sham: Ovarian intact, UW: Uterus Weight.

Table 2:

The effects of diets and E2 on lipid profiles and FBG in young and aged animals.

Parameters	Sham				OVX				Aged			
	SD	HFD	OIL	E2	SD	HFD	OIL	E2	SD	HFD	OIL	E2
TC (mg/dl)	81.4±2	121±3$^{\#\#\#}$	122±3	97.5±2§§§	87±2	140±3†††	138±1	109±2‡‡‡	100± 2	137± 4$^{\&\&\&}$	129± 5	129± 2
TG (mg/dl)	95±3	120±2$^{\#\#\#}$	121±2	107±3	106±2	145±3†††	129±4	122±3	113± 3	131± 4	130± 3	124± 5
FBG(mg/dl)	90±2	109±4$^{\#}$	110±3	102±2	110±7	147±2†††	154±2	120±3‡‡‡	140± 3	183± 3$^{\&\&\&}$	179 ±5	166± 7

Results are expressed as mean ± SEM., n=6-7 mice/group. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. Sham+SD. §§§P<0.001 vs. Sham+OIL. †††P<0.001 vs. OVX+SD. ‡‡‡P<0.001 vs. OVX+OIL. $^{\&\&\&}$P<0.001 vs. Aged+SD. E2: 17-β estradiol, FBG: Fasting Blood Glucose, HFD: High Fat Diet, OVX: Ovariectomy, Sham: Ovarian intact. SD: Standard Diet, TC: Total Cholesterol, TG: Triglyceride.