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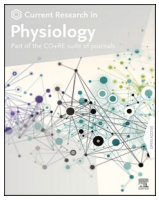

Metabolic effects of late-onset estradiol replacement in high-fat-fed ovariectomized mice

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ABSTRACT

Background: Decreased estrogen levels in postmenopausal women negatively impact metabolic health. It is known that estradiol (E2) replacement can reverse this condition. However, there is no consensus on whether the effects mediated by E2 depend on the starting time of E2 replacement after menopause. We aimed to investigate the effects of different onset E2 treatments on glucose tolerance and metabolic parameters in high-fat-fed ovariectomized mice.

Material and methods: Eight-week-old female C57BL/6J mice were divided into three groups: SHAM, OVX, and E2, to evaluate three different time points of E2 replacement after ovariectomy: early (after 4 weeks), intermediate (after 12 weeks), and late replacement (after 20 weeks). E2 groups received treatment through subcutaneous pellets.

Results: E2 replacement improved the parameters analyzed independently of the time since ovariectomy, reducing body weight gain and fat mass, as well as increasing the percentage of lean mass. Glucose intolerance, fasting insulin, HOMA-IR, and cholesterol levels were also reduced after treatment with E2. In the liver, there was a decrease in triacylglycerol (TAG) deposition, with no difference in the expression of SREBP1 and ERα proteins. In the muscle, there was a decrease in TAG deposition. In periuterine adipose tissue, there was an increase in the expression of SREBP1, FASN, and SCD, with no difference in the expression of ERα.

Conclusions: Our findings reinforce the critical role of E2 in regulating both glucose and lipid metabolism and indicate that E2 action on metabolic health was not dependent on time since ovariectomy for the parameters analyzed.

1. Introduction

Menopause is a physiological process characterized by the cessation of the production of sexual steroids by the ovaries and the end of a woman's reproductive life. The decline in circulating estrogen levels, especially estradiol, is followed by several changes in the female body, which become more pronounced in the context of obesity (Palacios et al., 2024). Among the changes, we can mention the development of cardiovascular (Hodis et al., 2015), neurodegenerative (Maioli et al., 2021) and metabolic diseases (Naftolin et al., 2019).

The development of metabolic imbalances occurs because estradiol plays an important role in insulin sensitivity, influencing glucose homeostasis and lipid metabolism (Camporez et al., 2013; Pereira et al., 2015). Decreased estradiol levels have been associated with weight gain and insulin resistance (Camporez et al., 2011; Araujo et al., 2023; Santos et al., 2024), redistribution of body fat and visceral obesity (Markova et al., 2024), in addition to sarcopenia (Pellegrino et al., 2022). Modifications in adipose tissue metabolism, becoming more lipolytic (Jeong et al., 2022) with reduced lipogenesis (Markova et al., 2024), lead to the accumulation of ectopic lipids which, in the liver, culminates in the development of metabolic associated fatty liver disease (Camporez et al., 2019; Talarico et al., 2023).

With the increase in life expectancy observed worldwide in recent decades (Kontis et al., 2017), there is an increase in the time that women will live after menopause. Therefore, therapeutic strategies are needed to prevent or treat these changes mentioned above. The adverse effects

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of estradiol reduction can be treated through hormone replacement therapy, through the administration of estrogens or the combination of estrogen and progesterone (Naftolin et al., 2019). Even though the indication of hormone replacement therapy is still controversial (Flores et al., 2021), clinical studies in postmenopausal women describe the beneficial effects of the use of hormone replacement therapy on insulin sensitivity (Salpeter et al., 2006), reducing plasma glucose and cholesterol levels (Kim et al., 2013, 2019), reducing waist circumference (Gambacciani et al., 2011) and visceral and total fat mass (Kleis-Olsen et al., 2024). Experimental studies using ovariectomized rodents, a translational animal model that mimics menopause (Venetsanaki et al., 2019), demonstrated that treatment with estradiol improved insulin sensitivity, in addition to reducing body weight and body fat in high-fat fed mice. These effects were associated with a reduction in ectopic lipid content, which, in turn, led to a reduction in the activation of protein kinase C (PKC) ε and θ (PKCε and PKCθ) in the liver and muscle, respectively, related to the process of insulin resistance (Camporez et al., 2013). Several models have also described other improvements in metabolic parameters (Araujo et al., 2023; Santos et al., 2024; Babaei et al., 2010, 2017; Kawakami et al., 2018).

The effects of estradiol on metabolic homeostasis occur mainly through the α isoform of the estrogen receptor (ERα). It was observed that ERαKO animals present marked weight and fat gain (Heine et al., 2000), in addition to changes in the translocation of GLUT-4 to the membrane, resulting in glucose intolerance (Barros et al., 2006). It is suggested that, as the years progress after menopause, the number of estrogen receptors in the female body decreases (Hodis et al., 2015). Thus, the question of whether there is an ideal window of opportunity to treat postmenopausal women with exogenous estrogens has received significant attention. The "window of opportunity hypothesis" or "timing hypothesis" consists of evaluating whether the benefits of estradiol treatment are related to the time interval since menopause.

Clinical studies evaluating cardiovascular and neuroprotective outcomes suggest that starting estradiol replacement in the early period of menopause is more beneficial (Naftolin et al., 2019; Daniel et al., 2006), with its effects in late menopause being uncertain (Pereira et al., 2015; Lee et al., 2023). Although it is relatively well established that estradiol promotes improvements in insulin sensitivity and energy metabolism, the vast majority of experimental studies have initiated the use of estradiol simultaneously with ovariectomy (Camporez et al., 2013; Araujo et al., 2023; Talarico et al., 2023) or one week later, considering a recovery period for these animals (Li et al., 2020; Riant et al., 2009; Saengsirisuwan et al., 2009). Few studies consider a relatively long window of time and evaluate metabolic parameters associated with obesity.

The present study was designed to evaluate whether estradiol-mediated effects on metabolic outcomes associated with obesity depend on the timing of treatment onset after menopause by using an experimental model.

2. Material and methods

2.1. Animals

Six-week-old female mice with C57BL/6J background were obtained from the Animal Care Facility of Ribeirão Preto Medical School, University of São Paulo. Animals were kept under controlled temperature (22 ± 2 °C) and humidity (50 ± 10 %) on a 12/12 h light/dark cycle (lights on at 6 a.m., lights off at 6 p.m.) and water and food *ad libitum*. All experimental procedures were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Ribeirão Preto Medical School, University of São Paulo (CEUA 1031/2021R1).

2.2. Ovariectomy

Eight-week-old female C57BL/6J mice underwent bilateral ovariectomy surgery (OVX), an experimental model that mimics menopause. Females were kept under anesthesia with isoflurane (Isoforine®), and two lateral cuts were made to remove the respective ovaries. At the end of the surgery, the animals received a dose of 0.05 mg/g of anti-inflammatory ketoprofen (Biofarm®) and 150 UI/g of penicillin (Penikel L.A.®) subcutaneously. A portion of the animals underwent sham surgery only (SHAM). Four days after surgery, a period for the animals to recover, all females were fed a high-fat diet (HFD) containing 45 % of calories from fat (D12451, Research Diets, NJ, USA) and had their body weight evaluated weekly. At the end of the experiments, the uterus was dissected and placed on filter paper, and its weight was measured to verify the success of the ovariectomy by uterus atrophy.

2.3. Experimental groups

To study the effects of different starting times of estradiol treatment after menopause, OVX mice were divided into two groups. Half of the OVX mice had subcutaneous pellets of 17β-estradiol implanted in the dorsal region (0.05 mg for 60 days, 35 μg/kg/day; Innovative Research of America®, Sarasota, USA) and released a constant dose of the hormone for 4 weeks (Araujo et al., 2023). The remaining animals received a vehicle. The time interval between ovariectomy and E2 pellet implantation was 4, 12, or 20 weeks. As a result, three experimental groups were formed for each time interval: SHAM, OVX and E2 in 4 wk group; SHAM, OVX and E2 in 12 wk group; SHAM, OVX and E2 in 20 wk group.

2.4. Glucose tolerance test (GTT)

After food restriction for 6 h, the mice received an intraperitoneal (i.p.) injection of glucose (1 mg/kg of body weight – 10 % dextrose). Blood samples for measuring blood glucose were taken by tail bleeding at 0, 15, 30, 45, 60, 90 and 120 min after injection. Also, homeostasis model assessment was calculated to determine the insulin resistance (HOMA-IR), following the formula: $\text{HOMA}-\text{IR} = ((\text{fasting glucose mmol/L}) \times (\text{fasting insulin } \mu\text{UI/mL}))/22.5$.

2.5. Magnetic resonance

The weight corresponding to lean mass and body fat was obtained through magnetic resonance imaging based on TD-NMR, using the LF50 minispec equipment (Bruker's, Massachusetts, USA).

2.6. Tissue lipid content

After a food restriction of 6 h, the liver and gastrocnemius muscle were collected for TAG extraction using the Bligh and Dyer method (Bligh et al., 1959), obtaining the lipid extract. The measurement was made with a specific TAG commercial kit (Bioclin®) following the manufacturer's instructions.

2.7. Oil Red O staining

Liver samples included in Tissue-Tek® were processed in the cryostat to obtain 12 μm thick sections. The sections were included on slides and stained with Oil Red O solution, a lipophilic dye that binds to the lipids present in the tissue and produces a reddish colour. Ten images of each animal were obtained under a microscope with a 20x magnification objective. The images were analyzed using ImageJ® software to obtain the Oil Red O positive area.

2.8. Measurement of insulin and cholesterol in plasma

After fasting for 6 h, the plasma was obtained and used to measure

fasting insulin using an enzyme-linked immunosorbent assay – ELISA (Mercodia®, Uppsala, Sweden). Total circulating cholesterol was also assessed using a specific commercial kit (Bioclin®, Belo Horizonte, Brazil). Plasma dosages were performed following the manufacturer's instructions.

2.9. Western blot

The following tissues were used for protein expression: liver and periuterine adipose tissue. Tissues were homogenized in RIPA buffer at 4 °C (1 % Triton-X-100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate and 2 mM PMSF) with Polytron PTA 20S homogenizer (Brinkmann Instruments). Tissue extracts were centrifuged at 12000 rpm at 4 °C for 20 min to remove insoluble material. After centrifugation, the Bradford method (BioRad) quantified the total protein content. Samples were treated with Laemmli buffer containing 200 mM DTT and 30 μg of total proteins solubilized from liver, muscle or adipose tissue fragments from mice. These samples were subjected to polyacrylamide gel electrophoresis (total extract). In each gel, there was a molecular weight marker with established values.

The proteins separated in the gel were transferred electrically to a PVDF membrane using a semi-dry transfer system (Bio-Rad) for 120 min. PVDF membranes were incubated with a blocking solution (5 % Bio-Rad skimmed milk, 10 mM Tris, 150 mM NaCl, and 0.02 % Tween 20) at 4 °C for 2 h to reduce nonspecific binding of proteins to the membranes.

The membrane was incubated with ERα antibody (Abcam, Cambridge, UK). To evaluate the lipid synthesis pathway, the membranes were incubated with the following antibodies: SREBP1 (Sterol Regulatory Element-binding Protein 1), SCD1 (Stearoyl-CoA Desaturase) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and FASN (Fatty acid synthase) (Cloud-Clone, Corp., Katy, TX, USA). All membranes were incubated with GAPDH (Santa Cruz Biotechnology, Inc., Santa

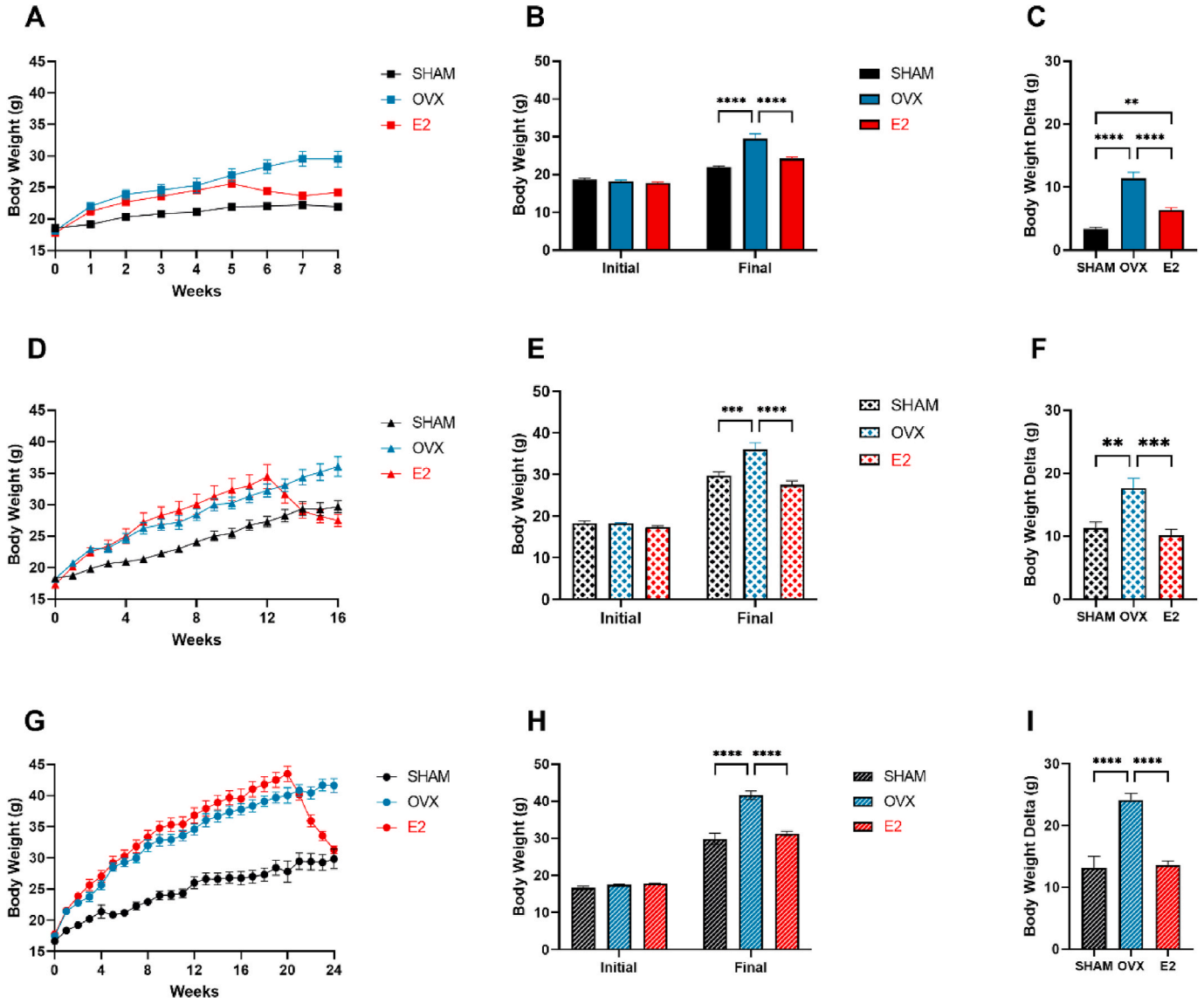

Fig. 1. wt gain curve data over the weeks of treatment (A, D and G), initial and final weight (B, E and H) and body delta, obtained by the difference between the initial and final weight (C, F and I). In A-C, data from SHAM (n = 9), OVX (n = 10) and E2 (n = 12) group 4 wk. In D-F, SHAM (n = 9), OVX (n = 10) and E2 (n = 12) from group 12 wk. In G-I, SHAM (n = 10), OVX (n = 11) and E2 (n = 12) from the 20 wk group. A, B, D, E, G and H: Significance between groups was determined by two-way ANOVA followed by Tukey's multiple comparisons test. C, F and I: Significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Cruz, CA, USA) to control the amount of protein in the membrane.

These incubations were carried out with a blocking solution (3 % BSA instead of milk) for 12 h at 4 °C, and the concentration of each antibody was as suggested by the manufacturer. Then, the membranes were washed with the blocking solution in milk for 30 min. These membranes were incubated with the second antibody, conjugated to peroxidase, for 2h at room temperature and, shortly after that, with the solution for chemiluminescence detection as described in the commercial kit protocol (ECLPlus, Amersham). Light emission was detected and visualized using a photodocumentator (iBright® CL750, Thermo Fisher Scientific). The intensity of the bands was quantified by optical densitometry using the band intensity analysis program (ImageJ).

2.10. Statistical analyzes

The results were analyzed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA). The minimum number of samples per group was defined by an *n* sufficient to perform sample distribution analysis using the Shapiro-Wilk normality test. Statistical analyses were performed using one- or two-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The results were expressed as means ± SEM. The minimum acceptable significance level was $p < 0.05$.

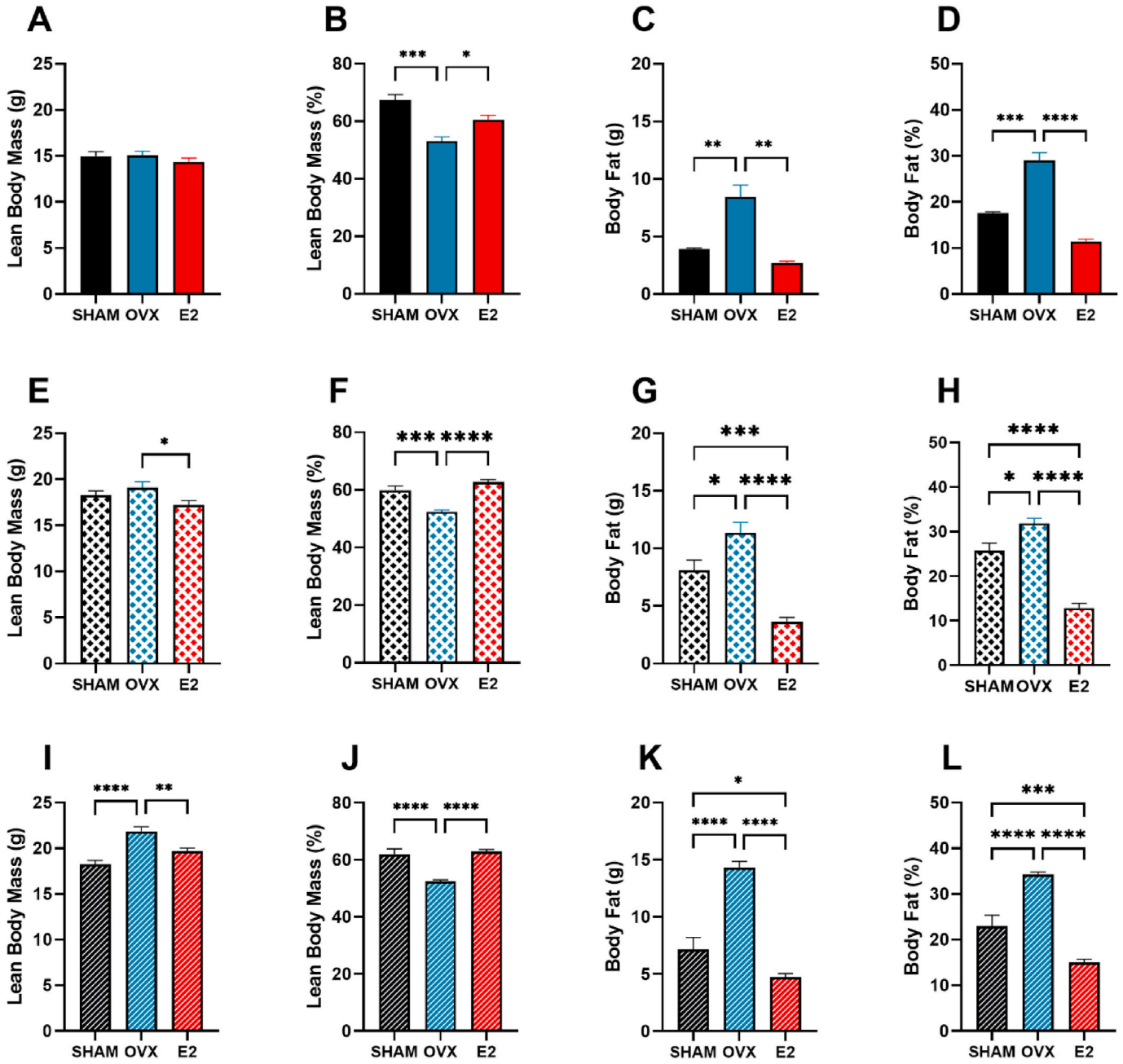

Fig. 2. Lean mass and body fat data obtained by body composition analysis using magnetic resonance imaging. The data presents absolute weight of lean mass (A, E and I), percentage of lean mass in relation to body weight (B, F and J), absolute weight of body fat (C, G and K) and percentage of fat in relation to body weight (D, H and L). In A-D, data from SHAM (n = 4), OVX (n = 6) and E2 (n = 3) group 4 wk. In E-H, SHAM (n = 10), OVX (n = 10) and E2 (n = 12) from group 12 wk. In I-L, SHAM (n = 10), OVX (n = 11) and E2 (n = 12) from the 20 wk group. Significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

3.1. Body composition

Body weight was monitored throughout the experiment (Fig. 1). There was no statistically significant difference in initial body weight between the SHAM, OVX, and E2 groups at different treatment times. However, at the end of the respective weeks of treatment, it is possible to observe that females in the OVX group have greater body weight, considering all time groups: 4, 12, and 20 wk. Also, it was seen that treatment with E2 could contain weight gain and reduce it, regardless of the start time, since the E2 groups had lower final body weight when compared to OVX and without statistical difference compared to the SHAM. These findings can be observed in the body weight curve data (Fig. 1A, D, and 1G), in which the E2 groups have a decrease in body weight in the last 4 weeks, concomitantly with the start of treatment; and in the initial and final body weight data (Fig. 1B, E and 1H). Now, observing the body weight delta of 12 and 20 wk groups (Fig. 1F and I), E2 groups have a smaller body weight delta compared to OVX ($p < 0.05$) and no statistical difference compared with SHAM ($p > 0.05$), that is, the absence of estradiol, represented by the OVX groups, led to more significant weight gain. However, regarding the body weight delta in the 4 wk group (Fig. 1C), E2 treatment did not fully reverse the change. This is evident as the E2 group exhibited a higher body weight delta compared to SHAM, a difference that was not observed in 12 and 20 wk groups.

Using magnetic resonance imaging for body composition analysis, the absolute weights of lean mass and body fat were obtained (Fig. 2). Regarding the absolute weight of lean mass, there was no statistically significant difference between the 4 wk groups (Fig. 2A). In the 12 wk groups, a decrease in the lean mass weight of E2 females was observed when compared to OVX females (Fig. 2E). In the 20 wk groups, the same difference is found between E2 and OVX. Also, OVX has a higher lean mass weight than SHAM (Fig. 2I). However, when calculating the percentage of lean body mass, we observed that in the three treatment time groups, both E2 and SHAM groups had a higher percentage of lean mass than OVX (Fig. 2B, F, and 2J). The analysis of body fat data reinforces the beneficial effects of estradiol in late menopause concerning body composition. It was seen that the E2 and SHAM groups had less body fat than the OVX group at all three treatment times, 4, 12, and 20 wk. Furthermore, for groups 12 and 20 wk, E2 females have a lower amount

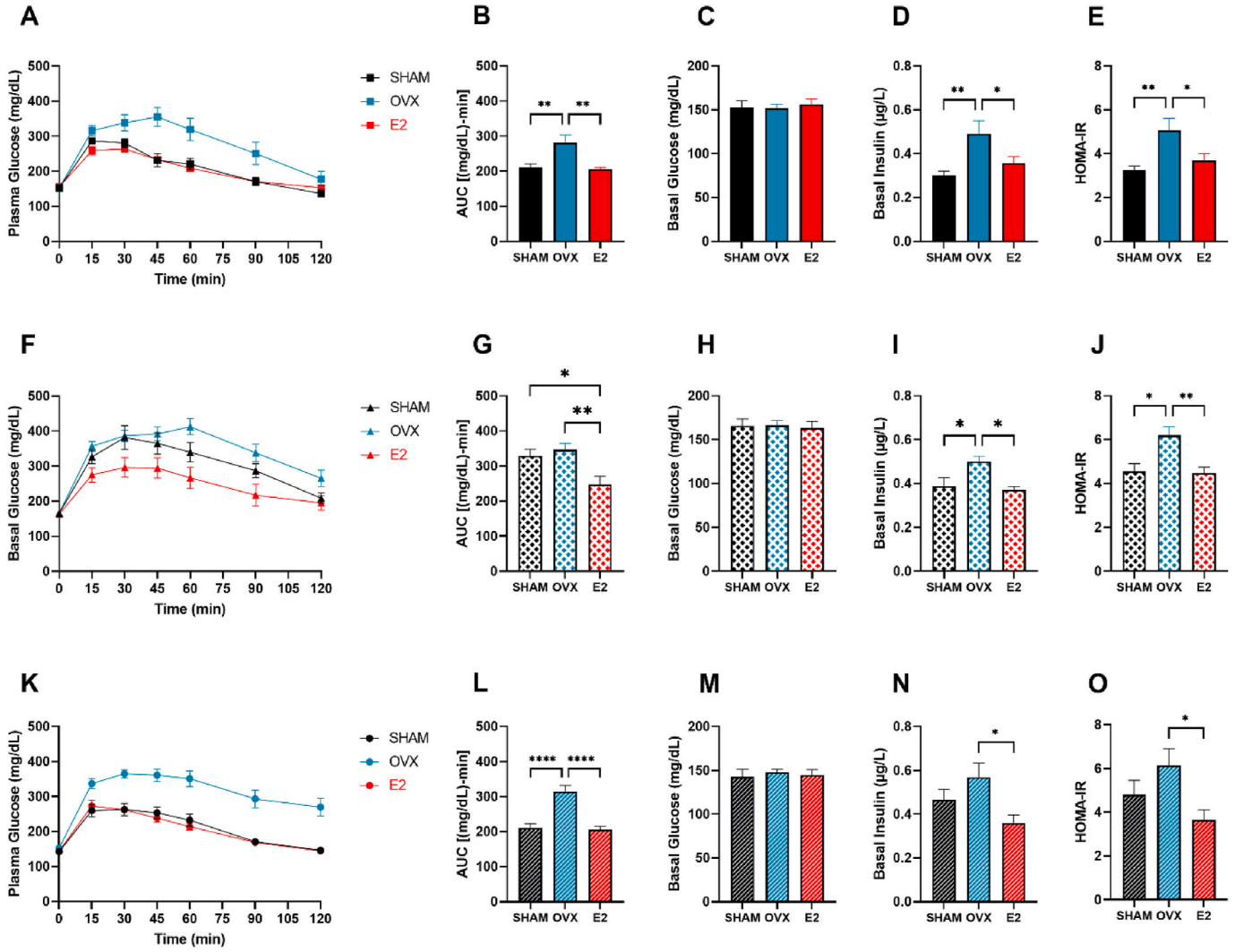

Fig. 3. Data obtained during the glucose tolerance test (GTT). The data presents a curve of blood glucose variation over time (A, F and K), area over the curve (B, G and L), basal blood glucose (C, H and M), basal insulin (D, I and N), and HOMA-IR (E, J and O). In 2A-2E, data from SHAM (n = 9), OVX (n = 10) and E2 (n = 11) group 4 wk. In F-J, SHAM (n = 10), OVX (n = 10) and E2 (n = 12) from group 12 wk. In K-O, SHAM (n = 10), OVX (n = 11) and E2 (n = 12) from the 20 wk group. A, F and K: Significance between groups was determined by two-way ANOVA followed by Tukey's multiple comparisons test. B-E, G-J, L–O: Significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$, with *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

(2C, 2G, and 2K) and percentage (Fig. 2D, H, 2L) of body fat compared to SHAM females.

3.2. Glucose tolerance

All animals were subjected to glycemic profile analysis using the GTT (Fig. 3). According to the glycemia curve over time (Fig. 3A, F, and 3K), it is possible to observe that, after the i.p. injection of glucose, the response pattern and glycemic return are different between the groups. For animals in groups 4 and 20 wk, E2 and SHAM females have a similar response profile to each other, as demonstrated by the lower glycemic curve compared to the OVX. However, for the 12 wk group, only the E2 group has lower glycemic levels. These differences become even more evident and reinforced when analyzing the area under the curve (AUC) (Fig. 3B, G, and 3L). There was no statistically significant difference in basal blood glucose (Fig. 3C, H, and 3M). These data suggest that estradiol replacement positively influences glucose metabolism, leading to lower glycemic levels in the treated groups, regardless of the time window for starting treatment.

Regarding basal insulin levels, in the 4 and 12 wk groups, E2 females have lower basal insulin levels than OVX and OVX have higher levels than SHAM group (Fig. 3D and I). For the 20 wk group, there is only a difference between the E2 and OVX groups, with females treated with estradiol having lower circulating insulin levels during fasting (Fig. 3N).

We used the HOMA-IR index to evaluate insulin resistance (Fig. 3E, J and 3O). The E2 groups showed a significant decrease at all treatment times compared to the OVX groups, suggesting that estradiol treatment reduces insulin resistance during early, intermediate, and late menopause. There is a statistical difference between OVX and SHAM in the 4 and 12 wk groups.

3.3. Ectopic and plasma lipid content

Triglyceride content (TAG) was measured in the liver and gastrocnemius muscle. In the liver tissue, at all three treatment times, E2 group showed a lower concentration of TAG when compared to OVX and no difference compared to SHAM. Furthermore, OVX group presented higher content than SHAM (Fig. 4A, C, and 4E). The results of the quantification of triglycerides in muscle tissue are similar to those in the liver (Fig. 4B, D and 4F), indicating, once again, that the benefits of E2 in the deposition of ectopic triglycerides are independent of how long after ovariectomy, the replacement is started. Considering the three protocols performed, E2 and SHAM groups had lower TAG concentrations than OVX. Furthermore, for the 12 wk groups, E2 females had lower tissue lipid content than SHAM (Fig. 4D).

The content of intracellular lipids in liver tissue was quantified by color intensity using the Oil Red O staining method. At all three treatment times performed, treatment with E2 significantly reduced tissue lipid content compared to the OVX, equating the amount with that of the SHAM group (Fig. 5), in line with the colorimetric measurement showed in Fig. 4. Furthermore, it is possible to observe that the percentage of the area occupied by the lipid droplets increases progressively, taking into account the time of exposure to the HFD, with the 4 wk groups having the lowest percentage of the area (Fig. 5A–B), followed by the 12 wk groups (Fig. 5C–D) and, finally, the 20 wk groups (Fig. 5E–F). These data reinforce the tissue quantification findings that, even in the late phase after ovaeriectomy, estradiol continues to exert effects in reducing the deposition of ectopic lipids.

Cholesterol levels were measured at the end of each experimental period (4, 12, and 20 wk) from plasma obtained after a 6-h fasting. The concentrations obtained through colorimetric testing demonstrate that the E2 and the SHAM groups had lower circulating cholesterol levels than the OVX groups at all three treatment times, 4, 12, and 20 wk, as seen in Fig. 6.

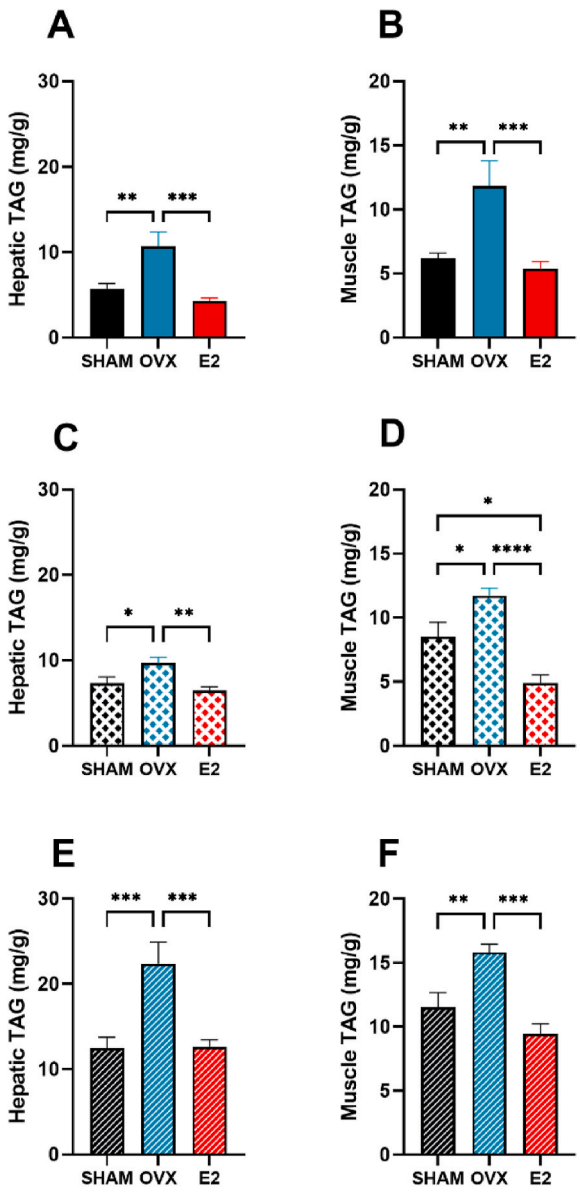

Fig. 4. The data shows the triglycerides concentration in the liver (A, C and E) and muscle tissue (B, D and F). In 1A-1B, data from SHAM (n = 9–10), OVX (n = 9–10) and E2 (n = 12) group 4 wk. In C-D, SHAM (n = 10), OVX (n = 10) and E2 (n = 10) from group 12 wk. In E-F, SHAM (n = 8–10), OVX (n = 10) and E2 (n = 10–12) from the 20 wk group. Significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$, with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.4. Protein content

After verifying the results of ectopic lipid deposition, we investigated the protein expression of ERα in the liver and periuterine adipose tissue, as well as proteins related to *de novo* lipogenesis, in the 20 wk groups (Figs. 7 and 8). ERα receptor expression did not differ between groups in the tissues analyzed (Figs. 7A and 8A). However, we observed an increase in the expression of sterol regulatory element-binding protein 1 (SREBP1), fatty acid synthase (FASN), and stearoyl CoA desaturase (SCD), proteins related to lipid synthesis, in the periuterine adipose tissue of E2 group when compared to OVX and SHAM (Fig. 7B–E). There was no statistically significant difference in the expression of the lipid synthesis marker in the liver (Fig. 8B–C).

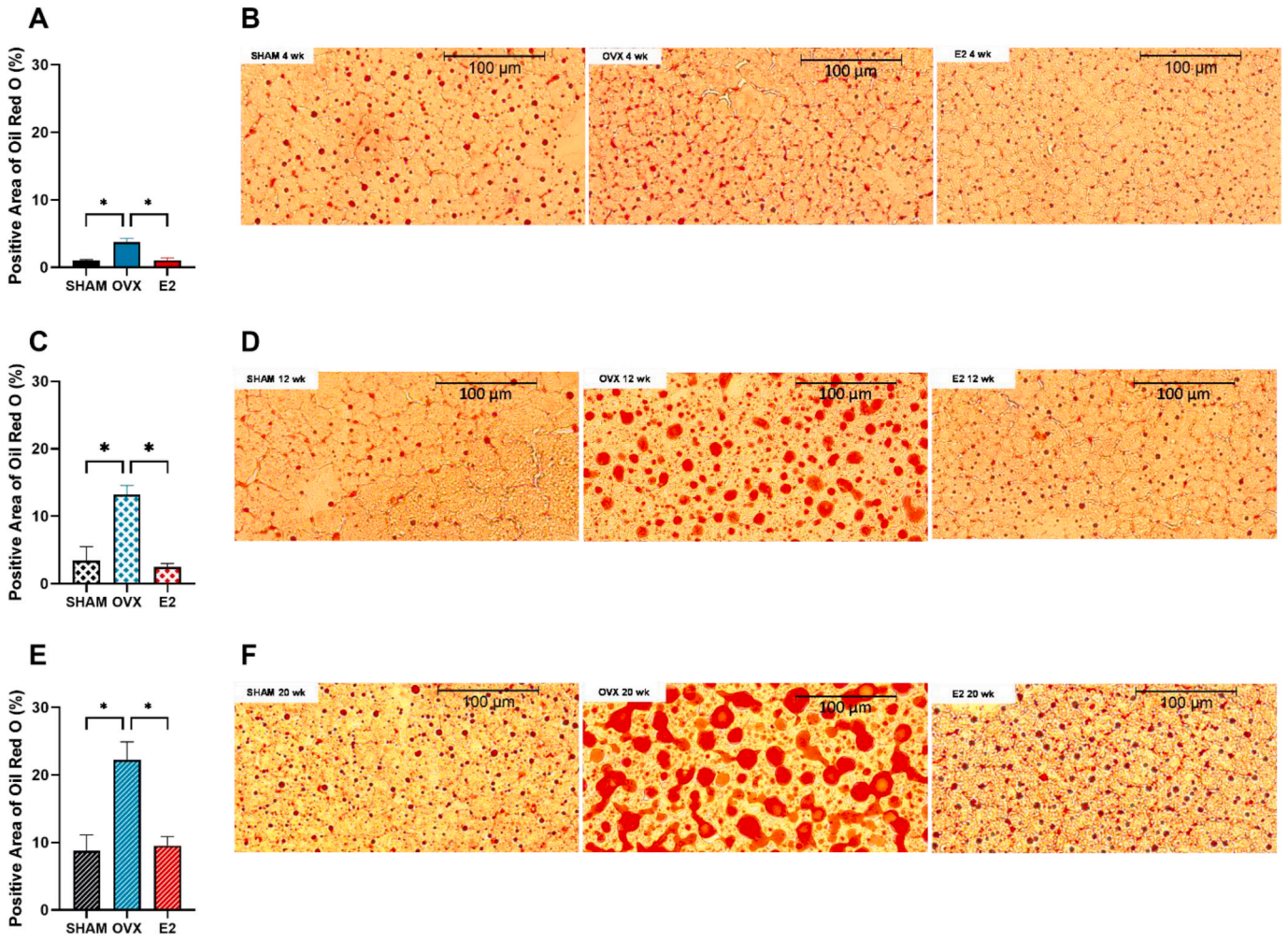

Fig. 5. Total area of lipid droplets in the liver. In A and B, quantification of the Oil Red O area and representative image of liver tissue, respectively, from groups 4 wk SHAM (n = 2), OVX (n = 3), E2 (n = 3). In C and D, quantification of the Oil Red O area and representative image of liver tissue, respectively, from groups 12 wk SHAM (n = 2), OVX (n = 2), E2 (n = 2). In E and F, quantification of the Oil Red O area and representative image of liver tissue, respectively, from groups 20 wk SHAM (n = 3), OVX (n = 3), E2 (n = 3). In A, C and E, significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparison test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$, with *$p < 0.05$.

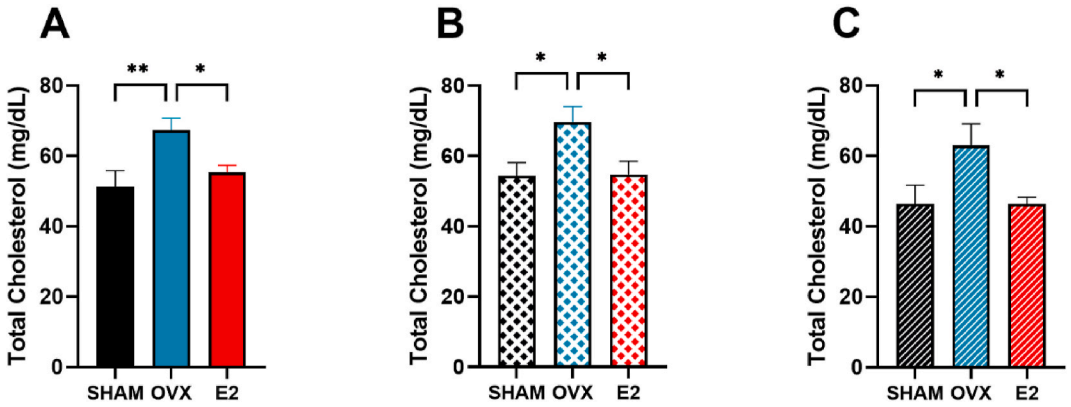

Fig. 6. Plasma total cholesterol measurement. In A, data from SHAM (n = 10), OVX (n = 11) and E2 (n = 11) group 4 wk. In B, SHAM (n = 10), OVX (n = 10) and E2 (n = 9) from group 12 wk. In C, SHAM (n = 10), OVX (n = 9) and E2 (n = 11) from group 20 wk. Significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$, with *$p < 0.05$, **$p < 0.01$.

4. Discussion

In the present study, we used different times to start estradiol replacement after surgical induction of menopause in an experimental model of ovariectomy and evaluated the effects on metabolic parameters. Several studies show that estradiol plays a vital role in metabolic

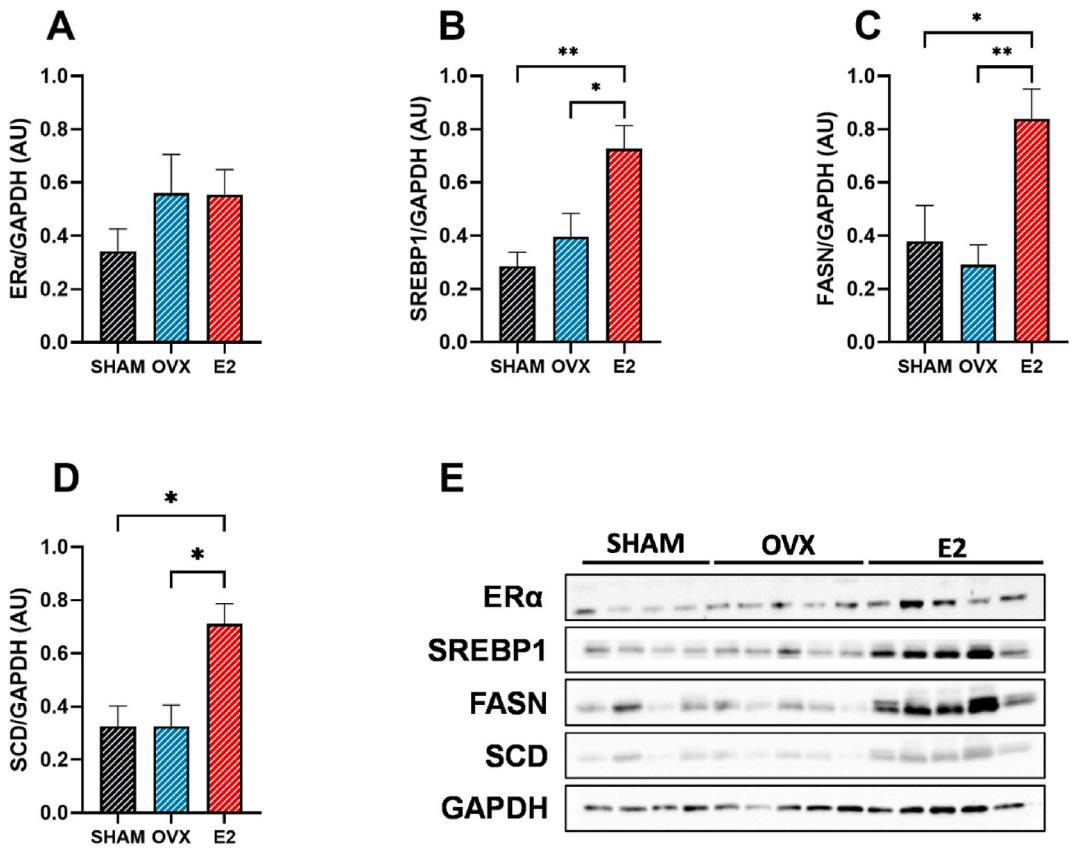

Fig. 7. Analysis of protein expression in periuterine adipose tissue by Western Blotting of group 20 wk SHAM (n = 4), OVX (n = 5) and E2 (n = 5). In A, quantification of ERα protein expression. In B, quantification of SREBP1 protein expression. In C, quantification of FASN protein expression. In D, quantification of SCD protein expression. In E, Western blot image of ERα, SREBP1, FASN, SCD and GAPDH,. Significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$, with *$p < 0.05$ and **$p < 0.01$.

homeostasis, and the decline in its circulating levels, as occurs during menopause, negatively impacts this balance (Ko et al., 2021). However, it is still unclear what the influence of the time window between the beginning of menopause and the start of estradiol replacement is. Our results showed that the beneficial effects of estradiol on weight and body composition, glucose metabolism and insulin resistance, hepatic steatosis, dyslipidemia, as well as effects on *de novo* lipogenesis, occur independently of the timing of hormone replacement therapy initiation in high-fat fed ovariectomized female mice.

It is already known that menopause leads to weight gain and body fat redistribution, favoring visceral fat deposition (Papadakis et al., 2018). This factor contributes to insulin resistance. Assessment of the animal's weight and body composition showed a marked positive energy balance for OVX females at three treatment times, 4, 12, and 20 wk (Figs. 1 and 2). This factor can be justified by the absence of ovarian production of estradiol, which is related to increased weight and body fat (Camporez et al., 2013; Santos et al., 2024; Talarico et al., 2023; Liu et al., 2024). It was previously demonstrated that OVX females have lower basal energy expenditure, in addition to reduced gene expression of oxidative metabolism markers, such as *Ucp-1, Cidea, and Prdm16*, in white adipose tissue, a possible pathway through which the energy expenditure is decreased (Camporez et al., 2013). Furthermore, the increase in weight and body fat in HFD-fed OVX females was related to increased visceral adipose tissue weight, in which the expression of *Fasn* and *Cpt1a*, genes related to the biosynthesis and oxidation of fatty acids, were reduced (Liu et al., 2024). In both studies, estradiol treatment was initiated immediately after ovariectomy. Our data reinforce these findings, since initiating estradiol replacement reduced body weight and the absolute and relative weight of fat content. However, contrary to literature data suggesting that estradiol in late menopause does not affect weight and body fat (Purnell et al., 2019), we showed that estradiol led to a reduction in body weight, as well as absolute weight and relative fat content regardless of the time to start the treatment.

In addition to the effects on body composition, we found that estradiol treatment reversed the glucose intolerance observed in the OVX groups and decreased basal plasma insulin levels (Fig. 3), suggesting improved insulin sensitivity, as observed in HOMA-IR data. Surprisingly, these effects were found in all groups treated with E2, regardless of the time of treatment onset. The benefits of estradiol in improving glycemic response in HFD-fed OVX females (Camporez et al., 2013; Liu et al., 2024) or in menopausal women (Pereira et al., 2015) are well described, considering the early phase of menopause. However, more conclusive data is needed taking into account a longer time window. Ramírez-Hernández et al. demonstrated that treatment with E2 after 20 weeks of ovariectomy did not change the HOMA-IR index in OVX rats, calculated from fasting blood glucose and insulin (Ramirez-Hernandez et al., 2023), suggesting no impact on insulin resistance. In another study using rhesus macaques, fasting insulin and the HOMA-IR index were only attenuated with treatment immediately after ovariectomy and, 24 months after surgery, these parameters had no changes (Purnell et al., 2019). In women, estradiol was observed to increase insulin-stimulated glucose utilization only in early postmenopausal women (Pereira et al., 2015), suggesting that the effects of estradiol were dependent on time postmenopausal rather than age (Pereira et al., 2015). In contrast, our results suggest that estradiol continues to exert its beneficial effects on glucose metabolism and insulin resistance even in late time period after ovariectomy, suggesting no change in glucose uptake signaling. It is essential to highlight that few

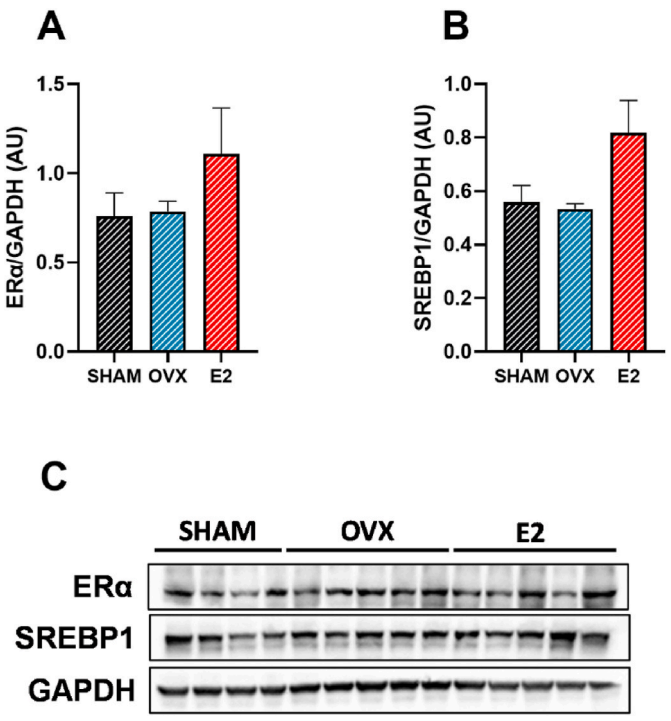

Fig. 8. Analysis of protein expression in the liver by Western Blotting of group 20 wk SHAM (n = 4), OVX (n = 5) and E2 (n = 5). In A, quantification of ERα protein expression. In B, quantification of SREBP1 protein expression. In C, Western blot image of ERα, SREBP1 and GAPDH. Significance between groups was determined by one-way ANOVA followed by Tukey's multiple comparisons test. Data presented as mean ± standard error of the mean (SEM). The minimum acceptable significance level was $p < 0.05$.

studies have compared the effects of different times of hormone replacement on glucose metabolism.

Menopause is also related to changes in the expression of hepatic (Jeong et al., 2022) and muscle (Abildgaard et al., 2018) lipid metabolism proteins. It has been described that ovariectomized female mice, generally associated with HFD consumption, develop hepatic and muscular insulin resistance and are prone to ectopic lipid accumulation (Camporez et al., 2013; Santos et al., 2024). OVX females showed increased TAG content in liver and muscle compared with SHAM and, interestingly, E2 treatment reduced TAG content regardless of the time of onset after ovariectomy (Fig. 4). In the liver, these findings were reinforced by quantification with Oil Red O (Fig. 5). Modifications in lipid metabolism inhibit glycogen synthesis, redirecting glucose to the lipogenic pathway, through the activation of proteins such as SREBP1 and its targets. However, there was no difference between groups in the content of this protein in the hepatic tissue (Fig. 8). Another related pathway is through diacylglycerol (DAG), a bioactive lipid mediator required for protein kinase C (PKC) activation. Ectopic TAG content is related to the increase in DAG and the subsequent activation of PKCε and PKCθ in the liver and muscle, altering the phosphorylation of the insulin receptor substrate IRS-1 to threonine and serine, respectively, resulting in a reduction in its ability to autophosphorylate on tyrosine and trigger insulin signaling (Petersen et al., 2016). Camporez et al. demonstrated that estradiol replacement immediately after ovariectomy decreased ectopic lipid accumulation, reducing DAG content in the liver and muscle, culminating in reduced activation of PKC corresponding to tissues and increased insulin sensitivity (Camporez et al., 2013). As estradiol acted similarly, reducing the accumulation of TAG in these tissues regardless of the time post-ovariectomy, our data suggest that the time window between the onset of experimental menopause and estradiol replacement does not influence this signaling pathway.

The total cholesterol profile in plasma was also analyzed, as the decline in estrogen levels resulting from menopause is associated with developing dyslipidemia (Malinska et al., 2021). Total cholesterol levels were reduced in all E2 groups, without differences with the SHAM (Fig. 6). Ovariectomy leads to an imbalance between the activity and synthesis of LDL and HDL, promoting arterial deposition (Ramirez-Hernandez et al., 2023), which leads to the formation of atherosclerotic plaques related to menopause (Hodis et al., 2015; Salpeter et al., 2006; Sriprasert et al., 2020). It is described that estradiol treatment is more effective in early menopause, and not in late menopause, in reducing cholesterol levels in postmenopausal women (Hodis et al., 2015; Sriprasert et al., 2020), ovariectomized rats and mice (Lee et al., 2023; Ramirez-Hernandez et al., 2023).

Changes in plasma lipid levels may be related to lipid metabolism in adipose tissue, which becomes more lipolytic because of the reduced estrogen levels (Jeong et al., 2022). As a result, the excess free fatty acids released contribute to the insulin resistance found in menopause (Yamatani et al., 2014). Estradiol replacement in late time period after ovariectomy increased the SREBP1, FASN, and SCD content in periuterine adipose tissue, suggesting an increase in the *de novo* lipogenesis pathway. Increased ERα expression was correlated with increased SREBP1 (Xie et al., 2022), which regulates the transcription of proteins involved in the fatty acid synthesis, such as FASN and SCD1.

In female OVX mice, decreased FASN expression was identified in subcutaneous and visceral adipose tissue (Macotela et al., 2009). Other studies demonstrated that ovariectomy decreases *Scd1* gene expression and increases it after estradiol treatment, indicating a relationship between decreased lipogenesis and tissue-specific insulin resistance (Markova et al., 2024). However, the opposite has also been described in aged non-OVX mice fed HFD and treated with estradiol (Al-Qahtani et al., 2017). Considering that SCD1 is an essential regulator in the control of lipid metabolism, it is important to highlight that a dual role of its activity in the progression of obesity has been described: its deficiency is related to the alleviation of the obese phenotype, and its overexpression protects from endoplasmic reticulum stress, related to insulin resistance (Sun et al., 2024).

Estradiol exerts its effects on energy metabolism mainly through ERα. It is suggested that, after menopause, the expression of ERα decreases (Hodis et al., 2015) or occurs a reversal of the ERα:ERβ ratio (Lv et al., 2022) over the years. However, we found no differences in ERα expression in the liver and adipose tissue during late menopause between the SHAM, OVX, and E2 groups (Figs. 7 and 8), suggesting that, even in the absence of the hormone for several weeks, the expression of the receptors does not change in our experimental model. Although it has been published that E2 replacement leads to an increase in ERα mRNA expression (Julien et al., 2019), in the present study the protein content of ERα was measured instead of mRNA. This detail could explain the discordance between the results.

In this work, we present the results of different times of initiation of estradiol treatment after ovariectomy on different components of metabolic health. Among the works in the literature that evaluate the time hypothesis, there are divergences regarding the species of the animal model, the use of different windows for treatment onset, and different treatment times, showing divergences about the benefits of estradiol treatment after a large window of time after the beginning of menopause. Our findings indicate that estradiol mitigates the adverse effects of experimental menopause in mice on glucose tolerance and metabolic parameters analyzed, even when treatment is initiated at later stages of experimental menopause. These data contribute to understanding the mechanisms by which estradiol influences metabolic homeostasis and highlight the importance of considering hormone replacement as a viable intervention for preventing metabolic disorders.

CRediT authorship contribution statement

Alessandra Gonçalves da Cruz: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Jessica**

Denielle Matos dos Santos: Investigation, Visualization. **Ester dos Santos Alves:** Investigation, Visualization. **Anne Raissa Melo dos Santos:** Investigation, Visualization. **Bruna Fantini Trinca:** Investigation, Visualization. **Felipe Nunes de Camargo:** Investigation, Visualization. **Guilherme Fancio Bovolin:** Investigation, Visualization. **João Paulo Camporez:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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