The effects of estradiol valerate and remifemin on liver lipid metabolism

Biao Jin\textsuperscript{a,1}, Wenjuan Wang\textsuperscript{a,1}, Wenpei Bai\textsuperscript{b}, Jing Zhang\textsuperscript{a}, Ke Wang\textsuperscript{a}, Lihua Qin\textsuperscript{a,*}

\textsuperscript{a} Department of Anatomy & Histology and Embryology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing, China
\textsuperscript{b} Department of Obstetrics and Gynecology, Beijing Shijitan Hospital, Capital Medical University, Beijing 100038, China

A R T I C L E   I N F O

Keywords:
Estradiol valerate
Remifemin
LXR
SREBP-1c
Ovariectomized rats

A B S T R A C T

To investigate the lipid metabolism dysregulation in the liver of ovariectomized (OVX) rats and effects of estradiol valerate (E) and remifemin (ICR) thereon, forty female Sprague-Dawley rats were randomly divided into sham-operated (SHAM), OVX, OVX+E, and OVX+ICR group. After 4 weeks' E or ICR treatment, serum estrogen, cholesterol, and triglyceride levels; lipid droplets in hepatocytes; hepatocyte morphology; and the expression of estrogen receptor \(\alpha\) (ER\(\alpha\)), liver X receptor (LXR), and sterol regulatory element binding proteins (SREBP) in the liver of the rats were assessed. OVX rats had significantly decreased serum estrogen levels, which significantly increased after treatment with E but not with ICR. Serum triglyceride levels and the amount of lipid droplets in hepatocytes increased after ovariectomy, and significantly decreased after E treatment. In addition, ICR treatment markedly increased serum triglyceride levels and lipid droplet size. No significant differences in the serum cholesterol levels were observed among the four groups. After ovariectomy, hepatocyte mitochondria became hypertrophic and misformed, which were reversed with E or ICR treatment. ICR-treated rats also showed endoplasmic reticulum disorganization. After ovariectomy, ER\(\alpha\) and LXR levels significantly decreased while SREBP expression increased. E treatment increased ER\(\beta\) and LXR levels while ICR treatment only increased LXR expression. E treatment decreased SREBP-1c levels, whereas SREBP-1c levels increased with ICR treatment. Treatment with E significantly reversed the ovariectomy-induced dysregulation of hepatic lipid metabolism, which was, however, exacerbated with ICR treatment. The effects of E and ICR on hepatocyte lipid metabolism may involve the regulation of LXR and SREBP-1c.

1. Introduction

Perimenopausal obesity is common in women undergoing involun-
tional changes due to the decrease in estrogen levels, which can alter lipid metabolism. Therefore, weight gain is one of the principal health problems in women aged 55–65 years (Nappi and Kook-Kierепа, 2012). Generally, the prevalence of obesity in women is higher than that in men; however, the underlying reason remains unclear. Changes in the levels of sex hormones during different physiological stages such as menarche, pregnancy, and menopause affect tissue fat deposition. For example, several studies have shown that during the perimeno-
pausal period, the deposition of abdominal adipose tissue increases (Davis et al., 2012). Perimenopausal obesity not only influences lipid metabolism but can also cause cardiac-related cerebrovascular disease, hypertension, and diabetes (Polotsky and Polotsky, 2010). Therefore, investigating the mechanisms underlying altered liver lipid metabolism in obese perimenopausal women as well as potential drug therapies targeting these mechanisms is important.

Estrogen is an important hormone with extensive physiological functions. Estrogen receptors (ERs) are steroid hormone receptors that modulate the physiological functions of estrogen (Weihua et al., 2000; Krege et al., 1998; Fan et al., 1999; Campbell-Thompson et al., 2001). ERs are expressed abundantly and comprise three subtypes: estrogen receptor \(\alpha\) (ER\(\alpha\)), estrogen receptor \(\beta\) (ER\(\beta\)), and G protein-coupled estrogen receptor (GPER). ER\(\alpha\) is mainly expressed in the liver, an organ playing an essential role in lipid metabolism (Mowa and Iwanaga, 2000; Denger et al., 2001). The distribution of ER\(\alpha\) and ER\(\beta\) in adipocytes varies between different organs. Further, only ER\(\alpha\) is known to inhibit fat deposition (Heine et al., 2000; Naaz et al., 2002). Some studies have shown that estradiol valerate (E) can effectively influence food intake in mammals. In addition, E plays an important role in maintaining lipid and glucose homeostasis. E has been shown to

**Abbreviations:** ER\(\alpha\), estrogen receptor; ER\(\beta\), estrogen receptor \(\beta\); GPER, G protein-coupled estrogen receptor; ICR, Remifemin; LH, luteinizing hormone; LXR, liver X receptor; NIH, National Institutes of Health; OVX+E, OVX with E treatment; OVX+ICR, OVX with ICR treatment; SREBP-1c, sterol regulatory element binding protein-1c; TG, triglyceride; TC, total cholesterol; TEM, transmission electron microscopy

**E-mail address:** qinlihua88@163.com (L. Qin).

* These authors contributed equally to this work.

Please cite this article as: Jin, B., Acta Histochemica (2017), http://dx.doi.org/10.1016/j.acthis.2017.06.004
reduce serum triglyceride levels in mice fed a high-fat diet (Faulds et al., 2012; D’Eon et al., 2005; Hewitt et al., 2004; Bryzgalova et al., 2008; Gao et al., 2006); however, the exact underlying mechanism is not completely understood. Other research has shown that ERs can inhibit lipoprotein lipase during the fasting state, and that an increase in serum estrogen levels can decrease the synthesis of triglycerides, as well as reduce the elongation of long-chain fatty acids family member 6 and fatty acid synthase factors (Iverius and Brunzell, 1988; Urabe et al., 1996). These factors play an important role in the aliphatic acid bio-synthesis pathway.

Liver X receptors (LXRs), which were discovered in 1994 (Willy et al., 1995), can regulate cholesterol absorption, synthesis, and excretion. There are two homologous LXR subtypes: LXRα and LXRβ. LXRα is not only highly expressed in the liver and involved in lipid metabolism, but is also found in adipose tissue, the prostate, intestines, lungs, kidneys, and macrophages (Zelcer and Tontonoz, 2006). LXRβ, on the other hand, is expressed throughout the body (Zelcer and Tontonoz, 2006). LXRs increase the expression of sterol regulatory element binding protein-1c (SREBP-1c), resulting in activation of lipogenesis (Horton et al., 2002). SREBPs are membrane-bound transcription factors, which participate in several aspects of lipid homeostasis in the liver (Ou et al., 2001). For example, SREBP-1c plays an essential role in the synthesis of triglycerides and phospholipids, whereas SREBP-2 mainly promotes the synthesis of cholesterol. The transcription of SREBP-1c can be activated by LXRs; therefore, inhibiting LXRs can downregulate the expression of SREBP-1c mRNA (Zhang et al., 2001). SREBP-1c can also be activated by insulin and inhibited by glucagon (Shimomura et al., 2000).

Remifemin (ICR) is the isopropanolic extract of the rhizome of the North American herb black cohosh (Cimicifuga racemosa (L.) Nutt. [CR], also known as raceme cohosh). Each tablet of ICR contains 1 mg of triterpene as the active ingredient, extracted from 20 mg of the crude drug. Black cohosh belongs to the Ranunculaceae family and grows in the eastern part of North America. It is used as a medicinal plant in Europe for more than a century. Black cohosh can be taken in normal doses for up to 6 months without showing any known side effects. Therefore, it is extensively used as an alternative for estrogen to treat perimenopausal problems. There is limited evidence that black cohosh may cause hepatic failure (Levitsky et al., 2005). The National Institutes of Health (NIH) reported that the results from a clinical trial on black cohosh did not confirm hepatotoxicity. Nevertheless, the NIH suggested that liver function should be monitored during clinical trials of black cohosh. Other research showed that black cohosh extract did not affect body weight or other clinical parameters; however, at doses of 667 and 2000 mg/kg it increased liver weight and serum alanine transaminase activity in women (Yun et al., 2015).

ERs affect liver lipid metabolism in rats (Faulds et al., 2012). For example, estrogen levels decreased and lipoprotein metabolism was altered accordingly in ovariec-tomized (OVX) rats (Han et al., 2014). At present, there are conflicting data regarding the therapeutic effect of ICR, and it is unknown whether the effects of ICR and E are mediated through common biological pathways. Therefore, in this study, we investigated whether and how ICR affects liver lipid metabolism. In addition, the effects of ICR were compared to those of E. We found that both E and ICR affected liver lipid metabolism through SREBP-1c. However, whether LXRs may play a role in this pathway remains unknown.

2. Materials and methods

2.1. Laboratory animals

Forty adult female Sprague-Dawley (SD) rats aged 9–10 weeks were obtained from the Department of Laboratory Animal Science of Peking University Health Science Center. All of the experiments were approved by the Laboratory Animal Welfare Ethics Committee of Peking University Health Science Center (approval number: LA2012-82). The rats were acclimatized for 1 week before the experiments and were fed a particle chow without beans (to exclude any potential effects of phytoestrogens) until the end of the experiments. The room temperature was kept at 25 °C, with a relative humidity of 50–55% and a 12-h/12-h light/dark cycle. The rats were allowed access to food and water ad libitum during the experiments.

2.2. Laboratory reagents and instruments

Mouse anti-rat LXRα (ab41902) was purchased from Abcam (Abcam, Cambridge, UK), rabbit anti-rat Phospho-SREBP-1c (Ser372) was purchased from CST, and rabbit anti-rat ERα antibody (SC-542) and the immunohistochemical ABC kit (SP-9001, SP-9002, PV6002) were purchased from Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd. (Beijing, China). ICR was purchased from Schaper & Brümmer GmbH & Co. KG (batch number 063471; Salzgitter, Germany). Each ICR tablet contained 20 mg of the crude drug, which was extracted with 40% isopropyl alcohol to yield an average of 2.5 mg of dried extract. E (1 mg active drug per tablet) was obtained from Bayer HealthCare Co., Ltd. (batch number 026A11; Guangzhou, China). Further, an ultrathin microtome (model 1900; Leica Biosystems, Wetzlar, Germany) and an Olympus BX51 microscope (Olympus, Tokyo, Japan) were used in this study.

2.3. Ovariectomized rat model

Forty rats were randomly divided into 4 groups: a SHAM, OVX, OVX with E treatment (OVX + E), and OVX with ICR treatment (OVX + ICR) group. Each group contained 10 rats. All of the rats, except those in the SHAM group, were bilaterally ovariectomized under aseptic conditions. Briefly, anesthesia was induced with an intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg) and a median abdominal incision of a length of 2–3 cm was made. The abdominal cavity was opened and the uterus was ligated using a forceps and filament. The ovaries were removed and the incision was sealed. The rats in the SHAM group underwent sham surgery. Briefly, after anesthesia induction with 1% pentobarbital, the pelvic cavity was opened without subsequent removal of the ovaries. The incisions were sealed thereafter. Starting from the third day after ovariectomy, vaginal smears were collected for up to 10 days to assess whether the surgery had been successful.

2.4. Drug administration

Two weeks after being ovariectomized, the rats were administrated the respective drugs by oral gavage between 8:00 AM and 9:00 AM each day for four weeks. The SHAM and OVX groups were administered 10 mL/kg normal saline. The OVX + E rats were administered 0.8 mg/kg E and the OVX + ICR rats were administered 60 mg/kg ICR. The doses of E and ICR were adjusted according to the daily body weight, and were prepared as previously reported (Xiao-yan et al., 2011).

2.5. Serology

Serum samples were collected from all of the rats in the four groups and the triglyceride (TG) and total cholesterol (TC) were measured using a Hitachi 7600-100 clinical analyzer (Roche, Japan, Department of Laboratory Medicine, Peking University Third Hospital, Beijing, China). Serum estrogen levels were measured using a radio-immunoassay (RIA) kit (R066, Solarbio Science and technology co., Ltd, Beijing). Testing steps were carefully carried out in accordance with the instructions in it.
2.6.2. Staining with hematoxylin and eosin (H & E) and Oil Red O

The samples were frozen and sliced using an ultrathin microtome PB (4 °C) containing 30% sucrose until they had sunken completely. The liver slices were subsequently soaked in 4% PFA in 0.1 mol/L PB (4 °C) for 4–6 h. The samples were subsequently removed and were incubated in 0.1 mol/L PB (4 °C) containing 30% sucrose until they had sunken completely. The samples were frozen and sliced using an ultrathin microtome (model 1900; Leica Biosystems, Wetzlar, Germany).

2.6.2.1. Preparation of liver slices

After anesthesia induction, the heart of each rat was exposed and the aorta ascendens was intubated through the left ventricle and the right auricle. Blood was flushed rapidly using 200 mL of normal saline until the liquid flowing from the right ventricle was colorless. The cardiac tissues were subsequently perfused and fixed with 300 mL of 4% paraformaldehyde (PFA, pH 7.4) in 0.1 mol/L phosphate buffer (PB, 4 °C) for about 30 min. Liver tissue samples were dissected immediately and were placed in 4% PFA in 0.1 mol/L PB (4 °C) for 4–6 h. The samples were subsequently removed and were incubated in 0.1 mol/L PB (4 °C) containing 30% sucrose until they had sunken completely. The samples were frozen and sliced using an ultrathin microtome (model 1900; Leica Biosystems, Wetzlar, Germany).

2.6.2.2. Staining with hematoxylin and eosin (H & E) and Oil Red O

The liver slices were flushed with water for 5 min, stained with hematoxylin for 5 min, and flushed with water again for 10 min. The samples were subsequently soaked in H & E staining solution for 3 s, flushed with water for 10 min, and soaked in 75% ethyl alcohol for 1 min. Next, the slices were stained with eosin solution for 5 s and soaked in absolute ethyl alcohol for 1 min, and the procedure was repeated once. Finally, neutral gum diluted in xylene was used to seal the slices. For the Oil Red O staining, the frozen slices were placed in 4% PFA solution and were washed twice with 60% isopropyl alcohol. The frozen liver slices were incubated in Oil Red O working solution (0684; Amresco, Cleveland, OH, USA) for 10 min after which 60% isopropanol alcohol was used for color separation. Glycerin solution (50% v/v) was used to seal the slices and images were acquired using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

2.6.3. Sample preparation for transmission electron microscopy (TEM) analysis

Liver samples were cut into small pieces (1 mm) and immersed in 2% buffered PFA and 1.25% glutaraldehyde (pH 7.4, 4 °C) for 2 h. Ultrathin sections were double-stained with 4% uranyl acetate and 0.1% lead citrate. The samples were visualized using a JEM-2100 electron microscope (JEOL, Tokyo, Japan) and images were acquired using brightness and contrast settings that were identical for each image and tissue section.

2.7. Western blotting

Small pieces of liver samples were dissected from each rat. Proteins were extracted and the protein concentration was quantified using a bicinchoninic acid protein assay kit (Applygen, Beijing). The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and were transferred to a nitrocellulose membrane (HATP00010, Millipore, USA). After blocking, the membranes were incubated with primary antibodies (rabbit anti-rat β-actin, 1:1000; mouse anti-rat LXRα antibody, 1:1000; and rabbit anti-rat p-SREBP-1c antibody, 1:800; Cell Signaling Technology, USA) at 4 °C overnight. The membranes were washed with 1 × Tris-buffered saline and Tween 20 thrice, and were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit, 1:2000; Applygen, Beijing). The proteins were visualized with the aid of Super ECL Plus Detection Reagent (P1010-100, Applygen, Beijing). The protein levels were normalized to those of β-actin (protein loading control).

2.8. Negative antibody controls for the Western Blots

The steps were almost the same as the procedures in the Western Blots above. After blocking, the membranes were incubated with normal serum instead of primary antibodies at 4 °C overnight. After being washed, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit, 1:2000; Applygen, Beijing). Exposure methods were as above. The protein levels were also normalized to those of β-actin (protein loading control).

2.9. Statistical analysis

The data are presented as the mean ± standard deviation (SD). The data were analyzed by one-way analysis of variance (ANOVA) and a Least Significant Difference (LSD) test using the SPSS software version 17.0 (SAS, Inc., Cary, NC, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Serology

The serum estrogen levels in the OVX rats decreased after 4 weeks’ treatment. They significantly increased after treatment with E (P < 0.05, Fig. 1A) and were higher than those in the SHAM rats. However, there was no significant difference in the serum estrogen levels between the OVX group and OVX + ICR group. The serum TG levels increased after ovariectomy, and decreased to levels similar to those in the rats in the SHAM group after treatment with E. The serum TG levels of the rats in the ICR group were significantly higher than those of the rats in the OVX group (Fig. 1B). However, no significant differences in the serum total cholesterol (TC) levels were observed among the four groups at the end of the treatment period (Fig. 1C).

![Fig. 1](image-url)
3.2. H & E staining and Oil Red O staining

The hepatocytes of the rats in the SHAM group showed a radial arrangement surrounding the central vein, forming the hepatic cord (Fig. 2A and E). The hepatocytes and hepatic cords of the OVX rats were arranged in a disordered manner (Fig. 2B and F). The number of lipid droplets in the rats in the OVX group was higher than that in those of the SHAM group (Fig. 3B and F). After 4 weeks of E treatment, the hepatic cord arrangements had an ordered morphology (Fig. 2C and G), and the number of lipid droplets decreased compared with that in the rats in the OVX group (Fig. 3C and G). The hepatic cords in the rats in the OVX+ICR group had a disordered morphology that was more severe than that in rats in the OVX group (Fig. 2D and H). In addition, the number of vacuoles in the hepatocytes increased and bulky vacuoles were observed in the rats in the OVX+ICR group (Fig. 3D and H).

3.3. Hepatocyte morphology analysis using TEM

Under physiological conditions, hepatocytes are relatively large and are polyhedral-shaped containing 68 faces (lateral and sinusoidal). In the SHAM group, the nuclei of the hepatocytes were large, round, and centrally located; chromatin was sparsely and lightly stained; however, some hepatocytes had two nuclei. The lipid droplets in the hepatocytes showed small, vacule-like structures (Fig. 4A). In the OVX group, TEM
revealed more vacuole-like structures in the hepatocytes and the vacuoles were larger (Fig. 4B). The number and size of the vacuole-like structures decreased in the rats treated with E (Fig. 4C). However, treatment with ICR increased the size of these structures and an accumulation of glycogen was observed around the vacuoles (Fig. 4D). After ovariectomy, the hepatocyte mitochondria were larger and their morphology was changed (Fig. 5A and B). No abnormal mitochondria were observed in the hepatocytes of the rats treated with ICR; however, an abnormally arranged rough endoplasmic reticulum (RER) was observed, which was absent in the hepatocytes of the rats in the other groups (Fig. 6D).

### 3.4. Western blot analysis

Negative antibody controls for the Western Blots showed that the antibodies (anti-mouse and anti-rabbit) had a high specificity. There were no specific bindings in the stripe (Fig. 7A and B). The expression of LXRα significantly decreased in the OVX rats ($P < 0.05$) compared with that in the SHAM rats. In the OVX+E and OVX+ICR rats, the expression increased to levels similar to those in the SHAM rats (Fig. 8A). Similarly, the expression of ERα significantly decreased after ovariectomy ($P < 0.05$), and increased in the OVX+E rats, albeit that the expression was significantly ($P < 0.05$) lower than that in the...
SHAM rats. Although not significantly, the ERα expression levels decreased in the OVX + ICR rats (Fig. 8B). Further, the expression of SREBP-1c significantly increased (P < 0.05) after ovariectomy and significantly decreased (P < 0.05) in the OVX+E rats. In contrast, in the OVX + ICR rats, SREBP-1c expression significantly increased and was significantly higher than that in the OVX rats (P < 0.05, Fig. 8C).

4. Discussion

Here we showed that ovariectomy resulted in increased serum TG levels, disarrangement of hepatocytes and hepatic cords, a significantly increased number of lipid droplets, and an increase in the volume of lipid droplets in rats. These results are indicative of a hepatic lipid metabolism disorder due to ovariectomy, which may be one of the causes of perimenopausal obesity. Previous studies showed that estrogen could prevent obesity and hyperlipidemia in OVX rats. For example, estrogen treatment of ER gene knock-out OVX rats did not affect/improve obesity and abnormal serum lipid levels, indicating that estrogen might regulate lipid metabolism mainly through ERs (Agirbasli et al., 2009; Barros and Gustafsson, 2011). In the present study, the number of lipid droplets in hepatocytes and serum TG levels significantly decreased in the OVX+E rats. Further, using TEM, we found that the average hepatocyte lipid droplet volume in the OVX+E rats was similar to that of hepatocytes in the SHAM rats, suggesting that estrogen deficiency resulted in disturbed hepatic lipid metabolism in the rats. Therefore, low estrogen levels may be a cause of perimenopausal obesity.

Estradiol valerate was contained in lots of preparations to treat menopausal complaints. It could be completely converted into natural 17β-estradiol and valeric acid, which was considered as an isoform or
derivative of estrogen. The pharmacokinetic and biotransformation of 17β-estradiol originating from estradiol valerate were no different from those of natural 17β-estradiol (Düsterberg and Nishino, 1982). In this study, estradiol valerate could significantly increase the serum estrogen and it also had many other therapeutical effects. The expression of SREBP-1c was decreased in the OVX + E rats, causing a decrease in the serum TG levels and number of lipid droplets in the hepatocytes. ERs modulate gene transcription mainly by binding to the special binding component of the estrogen response element (ERE), which is the so-called ER classical ligand-dependent pathway (Hall et al., 2001). ERs can also influence the interaction of proteins by selectively regulating reaction factors in the transcription process (Heldring et al., 2007). After the administration of a large dose of estrogen (10 mg/kg), ERα can reduce the accumulation of p300 in the SREBP-1c promoter region, thus reducing the expression of SREBP-1c (Han et al., 2014). In the present study, after administering a small dose of E (0.8 mg/kg) to the rats, we found that ERs regulated the transcription of SREBP-1c, indicating that the transcription of SREBP-1c was mediated through ERα. The role of LXR in this ERα and SREBP-1c pathway is unknown. In pancreatic β cells and skeletal muscles, E activates adenosine-monophosphate-activated protein kinase phosphorylation, which suppresses the expression of SREBP-1c and activates β-oxidation (D’Eon TM et al., 2005; Tiano and Mauvais-Jarvis, 2012). Further, E is known to decrease the expression of LXRs in adipocytes, hepatocytes, and pancreatic β cells (D’Eon et al., 2005; Tiano and Mauvais-Jarvis, 2012; Lundholm et al., 2004) thereby decreasing TG accumulation and expression of lipogenic genes. Other studies have shown that E can reduce lipogenic gene expression without decreasing LXRα expression (D’Eon et al., 2005; Bryzgalova et al., 2008). It is therefore suggested that ERs form dimers with LXRs to regulate the expression of SREBP-1c (Han et al., 2014). Further studies are required to ascertain if only ERs can influence the expression of LXRs. Collectively, studies above on lipid metabolism indicated that the expression of SREBP-1c was either related or not related to the expression of LXRs. However, in the present study, the expression of LXRs was significantly decreased while that of SREBP-1c was increased in the liver of OVX rats. As a metabolic organ, the liver is involved in several metabolic pathways. LXRs in hepatocytes not only regulate lipid metabolism but are also important regulators of glycometabolism. LXR activators can therefore significantly inhibit the expression of gluconeogenesis enzymes such as PEPCK, glucose 6-phosphatase, and fructose-1,6-bisphosphatase 1 (Stulnig et al., 2002a, 2002b), which suggests that LXRs are involved in the inhibition of gluconeogenesis. LXR activators have been shown to improve insulin sensitivity in Zucker rats (Agirbasli et al., 2009) but to reduce insulin sensitivity in OVX rats (Sun et al., 2016). This shows that the hepatic LXR expression affects lipid metabolism, glycometabolism, and immune response. Thus, LXRs had a high glycometabolic effect in the rats in the OVX group. However, the effects of LXR were more related to lipid metabolism in the OVX+E and OVX+ICR rats, resulting in decreased LXR expression and increased SREBP-1c expression. The present results do not prove whether LXRs are required for ERs to modulate the expression of SREBP-1c. The data, however, suggest that ERs can regulate SREBP-1c expression as an upstream factor and thereby influence hepatic lipid metabolism.

Fig. 6. TEM images showing the rough endoplasmic reticulum in the four rat groups. (A) SHAM group. (B) OVX group. (C) OVX E group. (D) OVX + ICR group. An abnormal morphology of the rough endoplasmic reticulum (RER) can be observed in the hepatocytes of the rats in the OVX + ICR group but not in those of the rats in the other groups. Black arrows indicate regular RER morphology; white arrows indicate abnormal RER morphology. Scale bar = 5 μm.

Fig. 7. Negative antibody controls for the Western Blots. (A) The secondary antibody was anti-mouse. (B) The secondary antibody was anti-rabbit. There were no specific bindings in the stripe.
Conflicting data exist on whether black cohosh can cause liver damage. Some cases of liver damage, including temporary autoimmune hepatitis (Pierard et al., 2009; Zimmermann et al., 2010) and even necrosis of hepatic lobules (Guzman et al., 2009), have been reported to be related to the use of black cohosh. Black cohosh can decrease the secretion of luteinizing hormone (LH) in OVX rats; however, this phenomenon has not been observed in perimenopausal women (Chung et al., 2007; Jacobson et al., 2001; Jarry and Harnischfeger, 1985; Nappi et al., 2005; Rachon et al., 2008; Reame et al., 2008; Seidlova-Wuttke et al., 2003). Although the results from the present study do not indicate the existence of a hepatotoxic effect of black cohosh, some clinical studies have shown such an effect (Thomsen and Schmidt, 2003; Thomsen et al., 2004). Nevertheless, to the best of our knowledge, thus far, studies of the molecular mechanisms underlying black cohosh-induced liver damage are lacking.

In the present study, after 4 weeks of ICR treatment, the expression of SREBP-1c downstream of LXR increased, indicating that ICR might influence hepatic lipid metabolism through the SREBP-1c pathway. Treatment with ICR did not affect the serum estrogen levels and hepatic ERα expression, suggesting that ICR did not have estrogenic effects but affected the SREBP-1c pathway. Studies in animals have shown that the extract of black cohosh rhizome has a negative feedback effect on the release of LH; however, the extract does not stimulate the uterus. This indicates that the extract of black cohosh rhizome selectively activates estrogen receptor modulators (SERMs) (Seidlova-Wuttke et al., 2003). Depending on the tissue type, SERMs have estrogenic or anti-estrogenic effects. SERM agonists and antagonists affect the activity of SERMs, the expression of ERα and ERβ, the relative expression levels of co-activators and co-repressors, the structure of target gene promoters, and signal pathways in cells (Shang and Brown, 2002; Jones et al., 1999). The aforementioned factors are related to the different effects of SERMs in different tissues. Although some studies showed that black cohosh could bind to ERs in vitro (Borrelli et al., 2003; Jarry et al., 2003) and increase ERβ expression in the uterus (Wuttke et al., 2008), other studies demonstrated that the estrogenic effect was not mediated by black cohosh (Borrelli et al., 2003; Beck et al., 2003; Zava et al., 1998; Burdette et al., 2003). Therefore, in the liver, black cohosh is more likely to have an anti-ER effect by affecting SERM activity. Although this effect does not change ER expression, it can change ER activity. E is an ER agonist and can, hence, increase ER activity. In this study, treatment with E attenuated the abnormal morphology of the hepatic cords and significantly reduced the number of lipid droplets. E also improved the serum estrogen levels and increased hepatic ERα expression, thereby most likely altering the expression of SREBP-1c and, thus, attenuating the abnormal hepatic lipid metabolism.

Further, ICR treatment attenuated the OVX-induced changes in mitochondrial morphology, indicating that black cohosh might have a protective effect on mitochondria. Indeed, others have shown that ICR significantly improved abnormal mitochondrial morphology in the lacrimal and submaxillary glands of OVX rats (Da et al., 2015). Furthermore, black cohosh can significantly enhance the activity of Cu-Zn superoxide dismutase in the rat liver and thus reduce oxidative stress/ 

$H_2O_2$ levels that cause hepatotoxicity (Campos et al., 2012). However, the lacrimal and submaxillary glands are different from the liver. Moreover, the OVX Wistar rats used in the previous study (Da et al., 2015) had also undergone unilateral nephrectomy, which is different from that used in the present study. Therefore, further research is warranted.

Here, the expression of LXRα was increased in the OVX + ICR rats. A previous study showed that LXRα inhibited the apoptosis of pathogenic bacteria-infected macrophages by upregulating anti-apoptotic factors such as Sp1 (Stulnig et al., 2002a). This shows that LXRα can inhibit the expression of inflammatory mediators, which may explain the attenuation of altered mitochondrial morphology in the OVX + ICR rats observed in this study. In addition, the RER showed signs of disintegration in the OVX + ICR rats, indicating that black cohosh might damage the RER. Previous work showed that black cohosh had a protective effect on the endoplasmic reticulum through anti-apoptotic mechanisms (Da et al., 2015). Although SD rats were used in that study as well and the method used to establish the model was the same as that used in the present study, the differences in protein composition and function between the lacrimal and submaxillary glands investigated in the previous study and the liver investigated in the present study should not be ignored. To the best of our knowledge, it is not known whether black cohosh has any effect on the endoplasmic reticulum. Although homeostasis within the endoplasmic reticulum is regulated effectively, any negative effects of black cohosh on hepatic lipid metabolism may most likely also compromise the endoplasmic reticulum.

This study showed that black cohosh disturbs hepatic lipid metabolism. Through the detection of relevant glucose tolerance indices, it was shown in a previous study that ICR could effectively reduce serum TG levels in OVX rats (Sun et al., 2016). However, in that study, SD rats received ICR treatment for 3 months, whereas the ICR treatment lasted 1 month in the present study. Therefore, to fully assess whether ICR may cause liver damage the effect of different doses and treatment durations of black cohosh need to be investigated.
5. Conclusions

Lipid metabolism is an important physiological process. Treatment with E can alter the expression of ERs and influence the expression of SREBP-1c. Although black cohosh does not have estrogenic effects, it can cause liver damage by increasing the SREBP-1c levels. LXRαs are important factors in liver lipid metabolism; however, whether they are also involved in the ERα − SREBP-1c pathway studied here needs further investigation. The main active ingredient in the rhizome of black cohosh is a terpene; however, the rhizome contains other biologically active ingredients such as alkaloids, flavones, and tannic acid. Determining the exact ingredient of black cohosh that causes disturbed hepatic lipid metabolism and the underlying mechanism requires further investigation.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant numbers: 81571399) and Beijing Natural Science Foundation (Grant numbers: 7162095). Both funding bodies were involved in the conduction and publication of this work.

References

Agbabhui, M., Agagbo, N.B., Orak, N., et al., 2009. Sex hormones and metabolic syn-
dromes in children and adolescents. Metabolism 58 (9), 1256–1262.
Beck, V., Unterritder, E., Krenn, L., et al., 2003. Comparison of hormonal activity (es-
trogen, androgen, and progestin) of standardized plant extracts for large scale use in hormone replacement therapy. J. Steroid Biochem. Mol. Biol. 84, 259–268.
Chung, D.J., Kim, H.Y., Park, K.H., et al., 2007. Black cohosh and St. John’s wort (Gyno-
Da, Y., Niu, K., Wang, K., et al., 2015. Comparison of the effects of estrogen and Cimicifuga racemosa on the lncrimal gland and submandibular gland in ovariec-
Shang, Y., Brown, M., 2002. Molecular determinants for the tissue specifici-
ity of SREBPs. J. Biol. Chem. 277 (35565), 2468–2474.
Stulnig, T.M., Oppermann, U., Steffenes, K.R., et al., 2002b. Liver X receptors down-
regulate 11 beta-hydroxysteroid dehydrogenase type 1 expression and activity. Diabetes 51 (8), 2426–2433.
Urabe, M., Yamamoto, T., Kashwagi, T., et al., 1996. Effect of estrogen replacement therapy on hepatic triglyceride, glucose, lipoprotein lipase and lipids including apolipo-


学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，
提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。
图书馆致力于便利、促进学习与科研，提供最强文献下载服务。