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Research Paper

Danzhi Qing'e (DZQE) activates AMPK pathway and regulates lipid metabolism in a rat model of perimenopausal hyperlipidaemia

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New Findings

- **What is the central question of this study?**
  Does Danzhi Qing'e (DZQE) regulate lipid metabolism and improve ovarian function in a rat model of perimenopausal hyperlipidaemia, and could this effect be mediated through the AMPK pathway?
- **What is the main finding and its importance?**
  We revealed that DZQE is a pharmacotherapy that could activate the AMPK pathway to improve ovarian function and lipid metabolism during perimenopause complicated with hyperlipidaemia syndrome in an animal model. Thus, this study provides a novel therapeutic option for treating perimenopausal syndrome and highlights the therapeutic potential of DZQE in perimenopausal rats.

Menopause is an important event in a woman's life. During perimenopause, accompanied by development of osteoporosis and dyslipidaemia, ovarian function gradually declines. Dyslipidaemia is a risk factor for cardiovascular disorders, cerebrovascular disease and breast cancer in postmenopausal women. All of these contribute to impairment of liver function, particularly fatty liver disease, because liver dysfunction is associated with ovarian dysfunction and hyperlipidaemia. The aim of this study was to define a therapeutic approach to improve ovarian function and attenuate lipid accumulation in order to prevent perimenopause-induced ovarian dysfunction and hyperlipidaemia. Four-week-old female Wistar rats were injected I.P. with 4-vinylcyclohexene diepoxide (4-VCD) and fed with a high-fat diet (HFD) to serve as a model of perimenopause complicated with hyperlipidaemia. The 4-VCD induces perimenopause, while the HFD causes hyperlipidaemia. Five days after administration of 4-VCD, the 4-VCD + HFD-treated rats were assessed daily for oestrous cycle stage by vaginal cytology. Rats were then assigned into groups, in which 2.5, 5.0 or 10.0 g kg^{-1} Danzhi Qing'e (DZQE) or estradiol valerate was administered intragastrically for 8 weeks. Expression levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestrogen and testosterone measured by enzyme-linked immunosorbent assay served as biomarkers for perimenopause and ovarian dysfunction. The expression levels of AMPK and acetyl-CoA carboxylase in the liver were determined with Western blotting, and aspartate aminotransferase and alanine aminotransferase were analysed using an automated biochemical analyser to examine liver function. The DZQE improved ovarian function by upregulating oestrogen and testosterone concentrations in serum

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and downregulating FSH and LH serum concentrations. Moreover, DZQE reduced serum concentrations of triglyceride, total cholesterol and low-density lipoprotein in a dose-dependent manner to regulate lipid levels during perimenopause. Furthermore, DZQE increased AMPK at both the transcriptional and translational levels and decreased the expression of *SREBP-1c* gene as well as its downstream target gene, fatty acid synthase. Danzhi Qing'e improved dyslipidaemia during menopause and also had an effect on liver function. Danzhi Qing'e is an effective Chinese herbal compound, which improves ovarian function and lipid metabolism in perimenopause complicated with hyperlipidaemia at least in part through the AMPK pathway.

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Introduction

Perimenopause is the time around menopause, starting from the appearance of a menopausal profile (serum hormone concentrations) until 1 year after the last menstrual period (Soules *et al.* 2001). Perimenopausal women usually suffer from ovarian dysfunction, with reduced production of oestrogen (E_2) and testosterone (T). They present with clinical manifestations such as, but not limited to, hyperlipidaemia and osteoporosis. Hyperlipidaemia is the most common and complicated symptom during perimenopause. Perimenopausal women are often diagnosed with hyperlipidaemia during routine physical examination (Agnoli *et al.* 2010; Ho *et al.* 2010; Berger *et al.* 2012; Thurston *et al.* 2013). A number of pathophysiological changes during menopause are associated with the function of the liver and mediate the pathogenesis of liver diseases. Loss of E_2 during menopause increases the risk of oxidative stress, mitochondrial dysfunction and hepatocyte damage. All of these contribute to impairment of liver function, particularly fatty liver disease. Given that liver dysfunction is associated with ovarian dysfunction and hyperlipidaemia, in the present study we examined whether Danzhi Qing'e (DZQE) could prevent liver dysfunction during perimenopause complicated with hyperlipidaemia.

Studies suggest that the E_2 produced by the ovaries during perimenopause contributes to the regulation of lipid metabolism. Hence, ovarian dysfunction during perimenopause leads to reduced production of E_2 and abnormal lipid metabolism. This underscores the need to improve ovarian function and attenuate the morbidity and mortality associated with lipid dysregulation in perimenopausal women (Sanada *et al.* 2002; Ho *et al.* 2010). Therefore, the discovery of drugs that are effective against ovarian dysfunction and dyslipidaemia will be a groundbreaking discovery in the pursuit of effective management to improve ovarian function and lipid metabolism (Yoshihisa *et al.* 2007). Both *in vivo* and *in vitro* experiments have proved the efficacy of natural compounds, suggesting that studies on natural compounds that improve oestrogen levels make substantial impact. Previous studies have demonstrated that the Chinese herbal medicine preparation Jiawei Qing'e Fang reduces hot flushes, improves menopausal symptoms and has a potential advantage in reducing triglyceride levels (Fu *et al.* 2016). Danzhi Qing'e is produced from Jiawei Qing'e Fang by adding an active ingredient. Available reports indicate that the DZQE formula improves the quality of life for menopausal women, especially for those with hot flush symptoms thorughout the menopausal period (Fu *et al.* 2016).

4-Vinylcyclohexene is a byproduct during manufacture of insecticides, flame retardants, rubber and plastic and is formed by dimerization of 1,3-butadiene (Cannady *et al.* 2003; Rajapaksa *et al.* 2007). Both *in vivo* and *in vitro* studies have provided evidence to show its role in inducing oocyte dysfunction (Sobinoff *et al.* 2010). Consistently, it has been proved that 4-vinylcyclohexene diepoxide (4-VCD) is an industrial chemical that causes ovarian toxicity (Smith *et al.* 1990; Kao *et al.* 1999). Currently, administration of 4-VCD and ovariectomy are the most common ways to simulate the pathological state of perimenopausal women. 4-Vinylcyclohexene diepoxide can selectively impair primary and primordial follicles and accelerate follicle apoptosis and, ultimately, causes premature ovarian failure (Rivera *et al.* 2009) without affecting other peripheral tissues, including the liver and spleen (Lohff *et al.* 2005; Muhammad *et al.* 2009). Importantly, circadian rhythms of oestrogen, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations in the 4-VCD-treated animal model are similar to the prognostic markers in perimenopausal women (Mayer *et al.* 2004; Muhammad *et al.* 2009). With regard to this, 4-VCD-induced premature ovarian failure is frequently used in basic research as a model

of perimenopausal syndrome (Muhammad *et al.* 2009). In the present study, treatment of Wistar rats with 4-VCD to stimulate perimenopause complicated with hyperlipidaemia served as the model of perimenopause.

Given that hyperlipidaemia is a complication of perimenopause, there has been a recent research spotlight and public interest in the regulation of lipid metabolism during perimenopausal syndrome. In this regard, 4-VCD-treated rats were fed with a high-fat diet to induce hyperlipidaemia. We examined the beneficial effect of DZQE in this rat model of perimenopause complicated with hyperlipidaemia by assessing the parameters associated with perimenopause and hyperlipidaemia. The measured parameters included FSH, LH, E_2, triglyceride, cholesterol (TC), low-density lipoprotein (LDL) and organ weight.

We also were interested in the effect of DZQE on signalling pathways pertaining to lipid handling specifically in the liver (Mayer *et al.* 2005; Greendale *et al.* 2011). AMPK is a heterotrimeric protein composed α (63 kDa), β (30 kDa) and γ subunits (37–63 kDa). After being activated by phosphorylation, it activates a large number of downstream target molecules to regulate lipid metabolism (López *et al.* 2010). Moreover, sterol regulatory element-binding protein (*SREBP*), is a downstream target gene of AMPK and is an important transcription factor that promotes fat synthesis (Espenshade 2006). SREBP-1c and SREBP-2 are the two main forms; SREBP-1c is mainly involved in the metabolism of fatty acids and triglycerides, whereas SREBP-2 is mainly involved in cholesterol metabolism. Hence, activation of the AMPK pathway is crucial in the treatment of hyperlipidaemia. Based on our findings, we suggest that DZQE alleviates the reduction in E_2 and T production during menopause. Additionally, we discuss the possible mechanism through which DZQE improves dyslipidaemia during menopause and determine its effect on liver function.

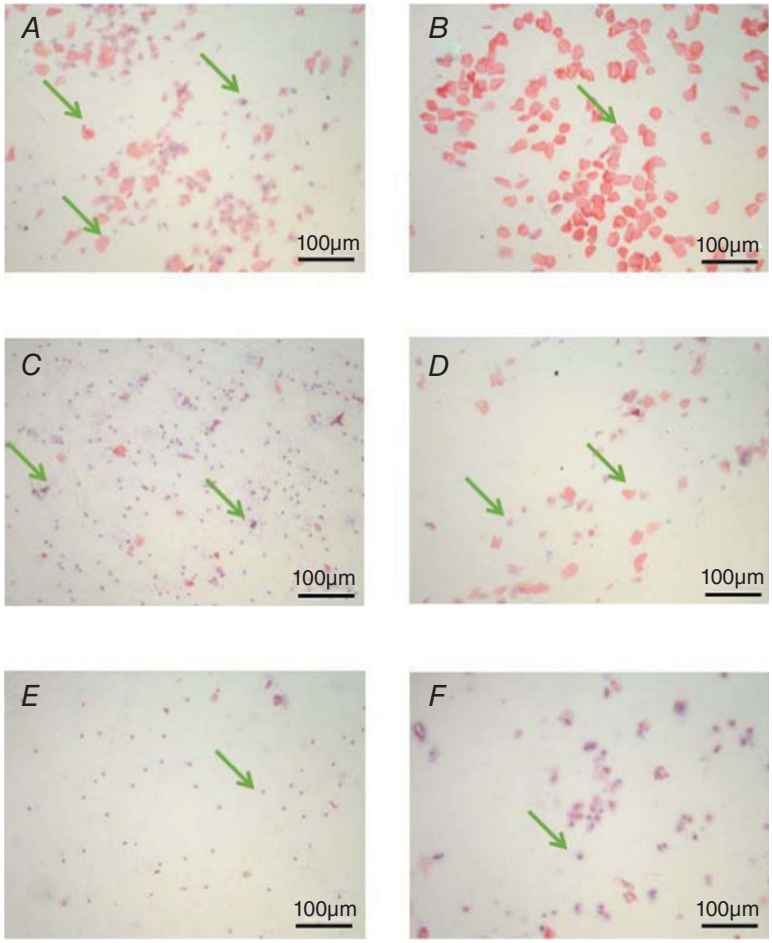

Figure 1. Representative vaginal cytology slides
A, normal rat in pro-oestrus. *B*, normal rat in oestrus. *C*, normal rat in late oestrus. *D*, normal rat in dioestrus. *E*, dioestrus rat in the 4-vinylcyclohexene diepoxide (4-VCD) group. *F*, 4-VCD-treated rat in late oestrus.

Methods

Ethical approval

All animal experiments were approved by the Institutional Animal Care and Use Committee, Tianjin University of Traditional Chinese Medicine and conformed to the *Guide for the Care and Use of Experimental Animals*. Three-week-old female Wistar rats (HuaFuKang biotechnology Co., Ltd, Beijing, China) were housed in plastic cages (four animals per cage) and maintained with a 12 h–12 h light–dark schedule at 22 ± 2°C. Water and pelleted food were available *ad libitum*. Animals were acclimated to the animal facility for 1 week (permit number: TCM-LAEC2015025).

Experimental protocol

4-Vinylcyclohexene diepoxide was used to induce follicular depletion, whereas a HFD was used to induce hyperlipidaemia, as previously reported. Four-week-old female Wistar rats were injected I.P. with 4-VCD dissolved in sesame oil at 80 mg kg^{-1} day^{-1} (0.01 ml g^{-1}, I.P.) for 15 days. Five days after administration, the 4-VCD-treated rats were assessed daily for oestrous cycle stage by vaginal cytology. For the vaginal cytology, a cotton wool tip was moistened slightly with saline and any excess was sharply flicked off. The rat was held around the upper part of the thorax and the ventral surface. The tip of the swab stick was inserted carefully into the rat's vagina to a depth of ~1.0 cm, with a rotating action of the swab and at an angle of about 45 deg to the animal's body. The vaginal secretions were smeared on a slide and stained with Haematoxylin and Eosin. Stained slides were examined to determine the number of epithelial cells present.

Those 4-VCD-treated rats ($n = 40$) with a terminated oestrous cycle were randomly divided into five groups. All the 4-VCD-treated rats ($n = 40$) were fed with high-fat diet, and the groups were as follows: 4-VCD + HFD ($n = 8$); DZQE, 2.5 g kg^{-1} ($n = 8$); DZQE, 5.0 g kg^{-1} ($n = 8$); DZQE, 10.0 g kg^{-1} ($n = 8$); and estradiol valerate (EV; 0.1 mg kg^{-1}, $n = 8$). The DZQE or EV was administered intragastrically for 8 weeks. Eight untreated female Wistar rats of the same age were used as normal control animals (Control, $n = 8$).

Preparation of DZQE

The DZQE consisted of extracts of *Eucommia*, *Anemarrhena*, *Salvia* and *Fructus psoraleae*, at ratios of 2:1:2:1, provided by the Department of Pharmacy, Institute of Chinese Medicine, Tianjin University of Traditional Chinese Medicine.

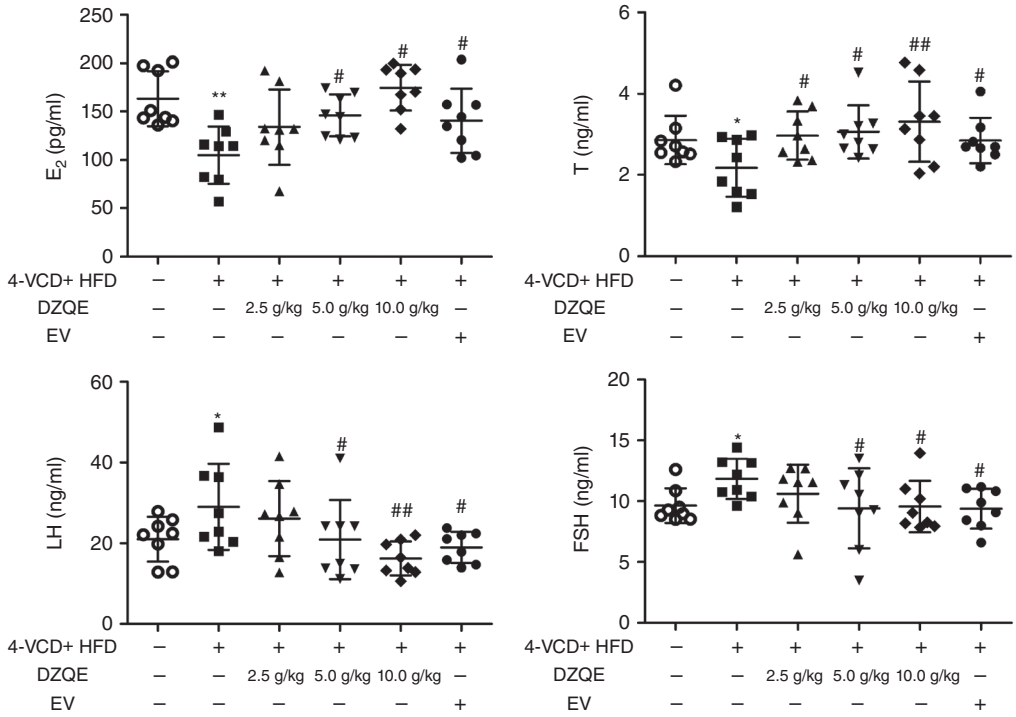

Figure 2. Changes of serum hormones in all six groups
Values are shown as means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* Control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* 4-VCD + HFD group. Abbreviations: DZQE, Danzhi Qing'e; E_2, estradiol; FSH, follicle-stimulating hormone; HFD, high-fat diet; LH, luteinizing hormone; T, testosterone.

Serum collection and tissue harvesting

At the end of the experiments, the rats were killed with chloral hydrate (5%, 6 ml kg^{-1}, I.P.). Then, blood samples were collected via abdominal aortic puncture. Serum was prepared by centrifugation of the collected blood at 447.2 g. for 20 min. Serum samples were stored at −80°C and were used to determine the levels of FSH, LH, E_2 and T with enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Serum lipids, aspartate transaminase (AST) and alanine transaminase (ALT) were analysed using an automatic biochemical analyser. The weights of the uterus, ovaries, liver, thymus, kidneys and brain were determined and were normalized to the body weight. The liver was cut into two portions; one was stored in 10% buffered formaldehyde and the other was washed with normal saline. The liver portions were then frozen with liquid nitrogen and stored at −80°C.

Histopathological analysis

Livers that were fixed in 10% buffered formaldehyde solution were gradually dehydrated, paraffin embedded and then sectioned at 5 mm thickness to be stained with Haematoxylin and Eosin. The pictures were taken with a Fuji digital camera (Tokyo, Japan) attached to a Nikkon trinocular microscope, model E200 (Tokyo, Japan). Images were submitted to two independent pathologists (Mao juan Guo and Bin Yu, Tianjin University of Traditional Chinese Medicine) for analysis and comparison between different groups. All procedures were followed according to good pathological anatomy practices.

qRT-PCR analysis

Total RNA was isolated from the liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was reverse-transcribed using the Super Script First Strand cDNA System (Invitrogen) according to the manufacturer's instructions.

The following primer sequences were used: *GAPDH*, forward (ATGATTCTACCCACGGCAAG) and reverse (CTGGAAGATGGTGATGGGTT); acetyl-CoA carboxylase (*ACC-α*), forward (AACAGTGTACAG CATCGCCA) and reverse (CATGCCGTAGTGGTTGAG GT); *AMPK*, forward (CTCGCCCAATTATGCTGCAC) and reverse (GGGAGAGTTCCACACAGCAA); oestrogen receptor-*α* (*Erα*), forward (CGAGGTGTACGTG GACAACA) and reverse (GTGATGCTCGACTGGCCA TA); oestrogen receptor-*β* (*Erβ*), forward (TGAACT ACAGTGTTCCCGGC) and reverse (GATGATTGG CAATGGGTCGC); lipoprotein lipase (*Lpl*), forward

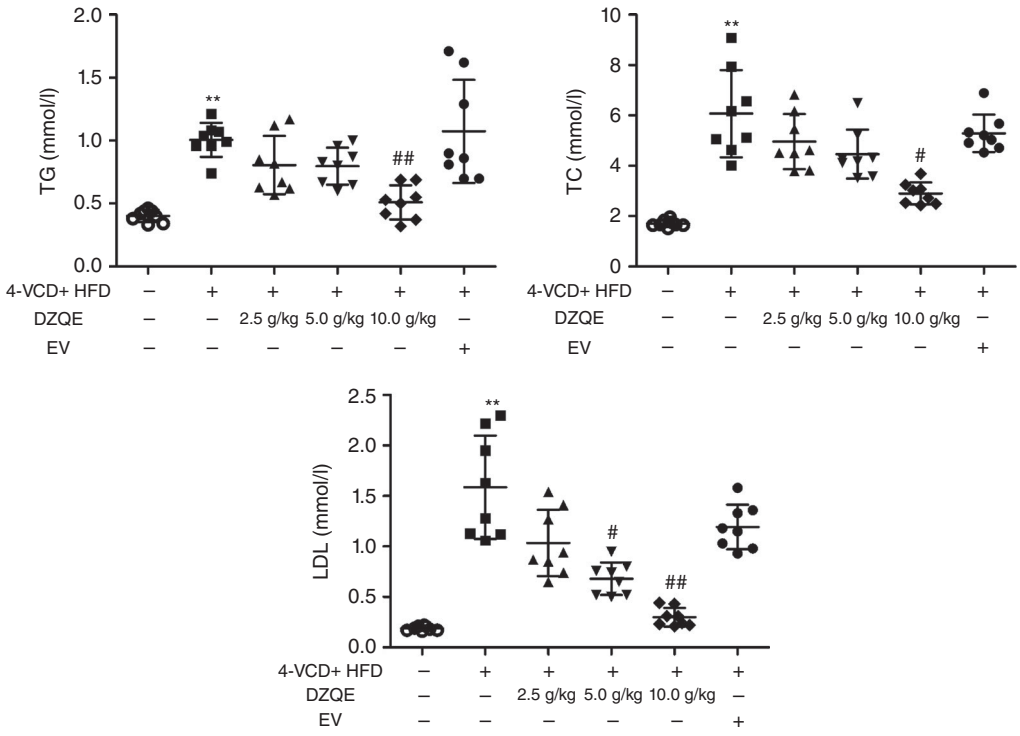

Figure 3. Changes of serum lipid in all the six groups
Values are shown as means ± SD. $^{**}P < 0.01$ *versus* Control, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* 4-VCD + HFD group. Abbreviations: LDL, low-density lipoprotein; TC, cholesterol; and TG, triglycerides.

(AACATTCCCTTCACCCTGCC) and reverse (TCTGA CCAGCGGAAGTAGGA); *SREBP-1c*, forward (TATC CCGTGTGCCTGGAAAC) and reverse (TGGATGC CAACCAGATTCCC); and fatty acid synthase (*FAS*), forward (GAGTATACAGCCACCGACCG) and reverse (AGTTGCACACCACAAGGTCA).

qRT-PCR was performed using SYBR Green PCR master mix (Applied Biosystems) with the 7900HT fast Real-time PCR system (Applied Biosystems). The relative levels of gene expression were represented as $\Delta C_t = C_t$ gene $- C_t$ reference, and the fold change of gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method (Nejat & Chervenak, 2010).

Western blotting assay

The liver tissue was homogenized in the lysis buffer using an ultrasound homogenizer at 50 Hz. The lysate was then centrifuged (1006.2 g). The protein concentration of the supernatant was measured using the bicinchoninic acid method. Proteins were loaded onto 8% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After blocking by non-fat milk, the membranes were exposed to primary antibodies overnight at 4°C. On the second day, the membrane was incubated with horseradish peroxidase-linked secondary antibodies for 2 h at room temperature and then visualized with chemiluminescence (ECL Blotting Analysis System; Amersham, Arlington Heights, IL, USA). Blotting on X-ray films was scanned and analysed using ImageJ software (Nationl Institutes of Health, Bethesda, MD, USA) and normalized to β-actin.

AMPK, p-AMPK, ACC and p-ACC antibodies were purchased from Cell Signaling Technology (Santa Cruz Biotechnology, Santa Cruz, CA, USA; #5831, #2535, #3676 and #3661, respectively), while β-actin antibody was obtained from santa cruz biotechnology (SANTA) SANTA Company (SC-47778).

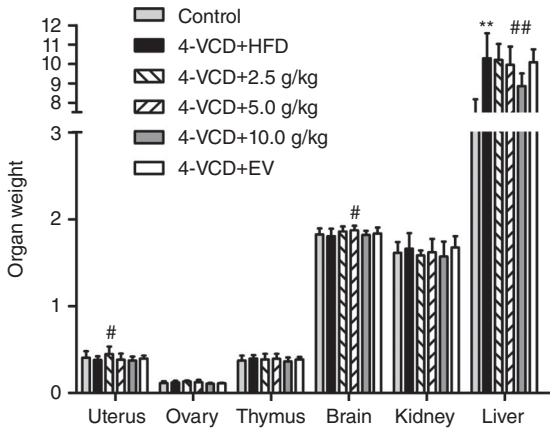

Figure 4. Effects of 8 weeks of DZQE treatment on organ weights of rats
Values are shown as means ± SD. $^{**}P < 0.01$ *versus* Control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* 4-VCD + HFD group.

Statistical analysis

All results were denoted as means ± SD. Statistical comparisons between different groups were performed by one-way ANOVA with Dunnett's multiple comparison *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

Results

Ovarian function

Four-week-old female Wistar rats were injected I.P. with 4-VCD dissolved in sesame oil at 80 mg kg^{-1} day^{-1} (0.01 ml g^{-1}, I.P.) for 15 days. Vaginal cytology showed that the oestrous cycle was present in female rats (Fig. 1). 4-VCD is mainly caused by primordial and primary follicles apoptosis, but there are some follicles that exist to simulate human perimenopausal.

Serum hormones

Compared with the Control group, serum concentrations of E_2 and T in the 4-VCD + HFD group were significantly reduced ($P < 0.05$ and $P < 0.01$, respectively). Compared with the 4-VCD + HFD group, concentrations of E_2 and T increased significantly in a dose-dependent manner in the DZQE 2.5, 5.0 and 10.0 g kg^{-1} groups. Compared with the Control group, serum concentrations of LH and FSH in the 4-VCD + HFD group were significantly increased ($P < 0.05$). Compared with the 4-VCD + HFD group, serum concentrations of LH and FSH were significantly reduced in the DZQE 5.0 and 10.0 g kg^{-1} groups ($P < 0.05$ and $P < 0.01$, respectively).

Estradiol valerate significantly upregulated the serum concentrations of E_2 and T, whereas the serum concentrations of LH and FSH were downregulated, compared with the 4-VCD + HFD group ($P < 0.05$; Fig. 2).

Serum lipid

Compared with the Control group, serum levels of triglyceride, TC and LDL in the 4-VCD + HFD group were significantly increased ($P < 0.01$). Compared with the 4-VCD + HFD group, DZQE 10.0 g kg^{-1} reduced serum concentrations of triglyceride, TC and LDL significantly ($P < 0.01$, $P < 0.05$ and $P < 0.01$). Estradiol valerate had no significant effect on serum lipid, compared with the 4-VCD + HFD group (Fig. 3).

Organ weights

There was no significant change in the weights of ovaries, thymus and kidneys among all the six groups. Compared

with the 4-VCD + HFD group, the weights of the brain and uterus in the DZQE 2.5 and 5.0 g kg^{-1} groups were significantly increased ($P < 0.05$, respectively); the weight of the liver in the DZQE 10.0 g kg^{-1} group was significantly increased ($P < 0.01$). Compared with the Control group, the weight of the liver in the 4-VCD + HFD group was remarkably increased ($P < 0.01$; Fig. 4).

The control group exhibited normal histology, which showed the liver cells arranged neatly and radially around the central vein. The hepatocyte nuclei were large and round, the cytoplasm was eosinophilic, and most cells were polygonal. Compared with the Control group, the 4-VCD + HFD group exhibited abnormal histological changes that were indicative of liver injury.

Liver function

Given that the liver is an important organ for metabolism and that liver weights of the 4-VCD + HFD group differed significantly from those in the Control group, liver function tests and liver pathological examinations were performed.

Compared with the Control group, the concentrations of ALT and AST in the 4-VCD + HFD group were significantly increased ($P < 0.05$ and $P < 0.01$, respectively). Compared with the 4-VCD + HFD group, the DZQE 2.5, 5.0 and 10.0 g kg^{-1} groups exhibited significantly lower concentrations of ALT and AST (Fig. 5).

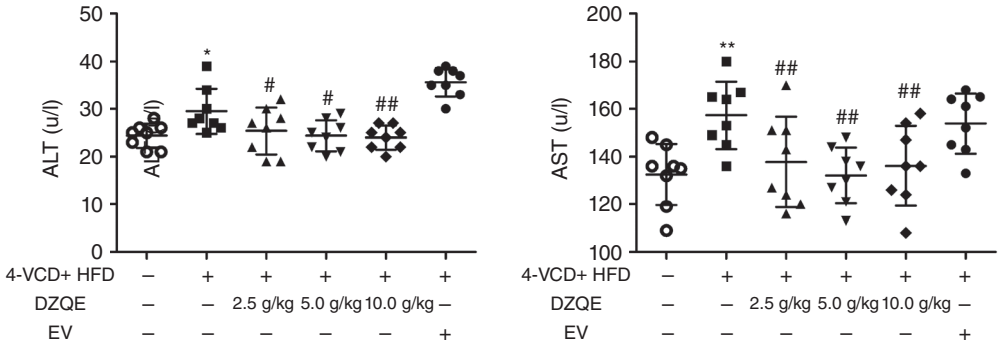

Figure 5. Liver function tests in all six groups
Values are shown as means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* Control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* 4-VCD + HFD group. Abbreviations: ALT, alanine aminotransferase; and AST, aspartate aminotransferase.

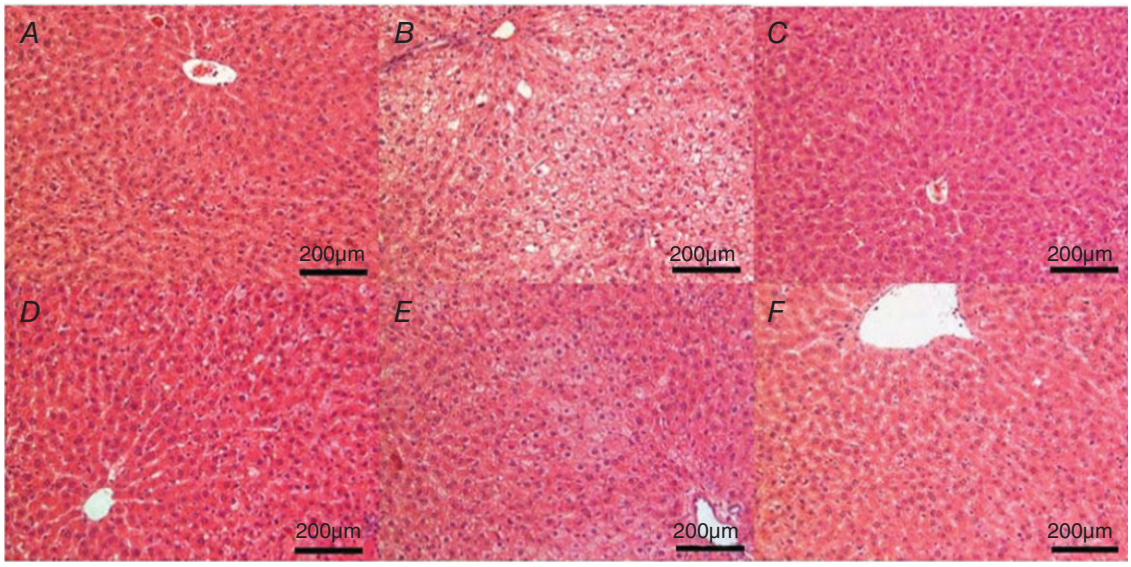

Figure 6. Representative histopathology slides
A, liver section of Control group (normal liver). *B*, liver section of 4-VCD + HFD group. *C*, liver section of DZQE 2.5 g kg^{-1} group. *D*, liver section of DZQE 5.0 g kg^{-1} group. *E*, liver section of DZQE 10.0 g kg^{-1} group. *F*, liver section of EV group.

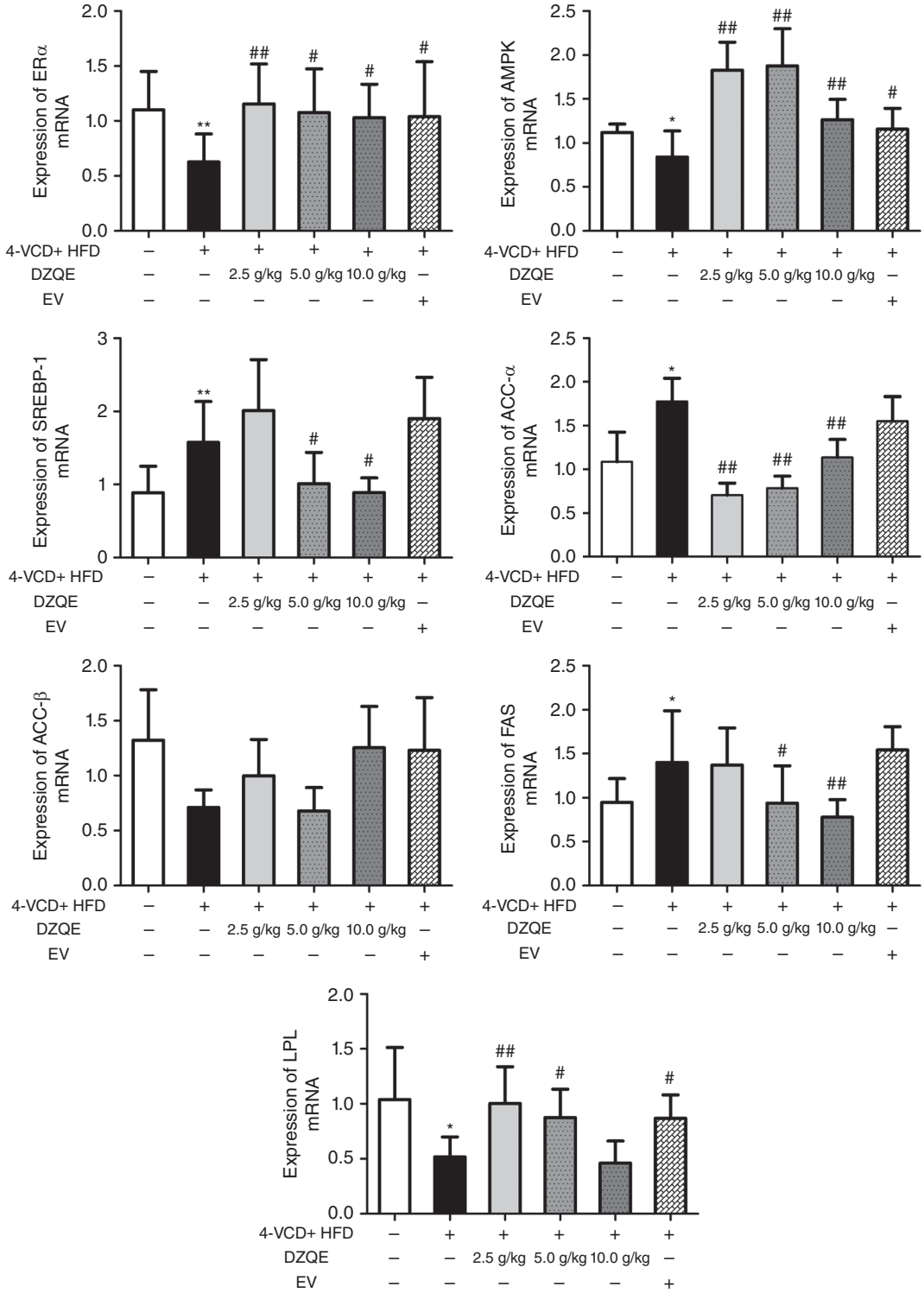

Figure 7. Changes of gene expression in the liver in all six groups
Values are shown as means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* Control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* 4-VCD + HFD group. Abbreviations: *ACC-α*, acetyl-CoA carboxylase *α*; *ACC-β*, acetyl-CoA carboxylase *β*; *AMPK*, AMP-activated protein kinase; *ERα*, oestrogen receptor-*α*; *FAS*, fatty acid synthase; *LPL*, lipoprotein lipase; and *SREBP-1c*, sterol regulatory element-binding protein-1.

Liver pathology

Compared with the 4-VCD + HFD group, the fatty degeneration of liver cells in the DZQE 2.5, 5.0 and 10.0 g kg^{-1} and EV groups was significantly reduced (Fig. 6).

Gene expression in the liver

Compared with the Control group, gene expressions of *ERα*, *AMPK* and *LPL* in the 4-VCD + HFD group were remarkably reduced, whereas levels of *ACC-α*, *SREBP-1c* and *FAS* were significantly increased ($P < 0.05$ and $P < 0.01$). Compared with the 4-VCD + HFD group, DZQE 2.5, 5.0 and 10.0 g kg^{-1} increased gene expressions of *ERα* ($P < 0.01$, $P < 0.05$, $P < 0.05$ respectively) and *AMPK* ($P < 0.01$, respectively) and reduced the expression of *ACC-α* ($P < 0.01$, respectively). Both 5.0 and 10.0 g kg^{-1} DZQE reduced the expressions of *SREBP-1c* ($P < 0.01$, respectively) and *FAS* ($P < 0.05$ and $P < 0.01$), whereas both 2.5 and 5.0 g kg^{-1} DZQE increased *LPL* expression ($P < 0.01$ and $P < 0.05$).

Estradiol valerate increased gene expressions of *ERα*, *AMPK* and *LPL*, compared with the 4-VCD + HFD group (Fig. 7).

Protein expression in the liver

Compared with the Control group, protein expressions of P-AMPK and P-ACC in the 4-VCD + HFD group were reduced, whereas SREBP-1 was significantly increased ($P < 0.01$). Compared with the 4-VCD + HFD group, DZQE increased expressions of P-AMPK ($P < 0.01$) and P-ACC 2.5 g/kg, 5.0 g/kg and 10.0 g/kg ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) and reduced expression of SREBP-1 ($P < 0.01$; Fig. 8).

Discussion

Ovarian dysfunction is a prognostic marker for perimenopause, which leads to the reduced production of oestrogen and testosterone and, ultimately, causes lipid accumulation (Vicennati *et al.* 2015). Administration of 4-VCD induces ovarian ageing that mimics the physiological and pathological conditions of women with premature ovarian failure by impairing original and primary follicles (Hoyer *et al.* 2001).

In this study, we observed that 4-VCD impaired primordial and primary follicles in ovaries of juvenile and adult rats. 4-VCD treated group didn't have oestrus (Fig. 1). Furthermore, we observed a lower E_2 but higher FSH and LH concentrations in the 4-VCD + HFD group compared with those in the Control group ($P < 0.05$). The serum concentration of E_2 increased, whereas serum concentrations of FSH and LH decreased in the 5.0 and 10.0 g kg^{-1} DZQE groups compared with those in the 4-VCD + HFD group. These results indicate that DZQE is able to improve ovarian function and correct hormonal disorders during perimenopause. Additionally, androgens

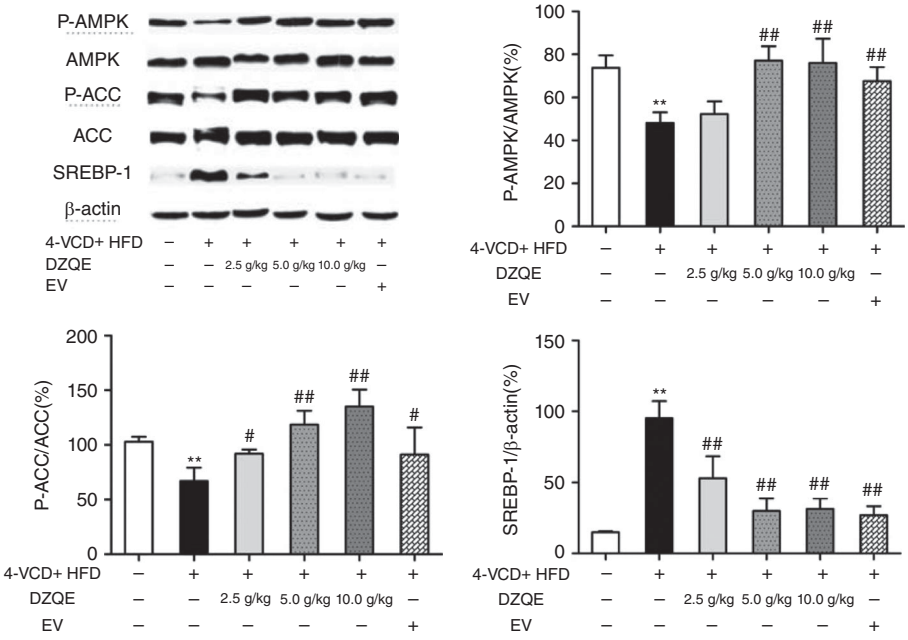

Figure 8. Changes of protein expression in the liver in all six groups
Values are shown as means ± SD. $^{**}P < 0.01$ *versus* Control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* 4-VCD + HFD group. Abbreviations: AMPK, ACC, acetyl-CoA carboxylase; AMP-activated protein kinase; and SREBP-1, sterol regulatory element-binding protein-1.

also play an important role in perimenopause. Hence, the observation that DZQE could elevate testosterone levels in 4-VCD-induced ovarian dysfunction corroborates the contention that DZQE has a therapeutic significance in improving ovarian function during perimenopause.

Hyperlipidaemia is a common symptom in perimenopausal women and has been associated with ovarian dysfunction during perimenopause (Burger *et al.* 2008). In light of this, in this study we also examined whether DZQE could improve lipid regulation after 4-VCD + HFD administration. The observation that serum concentrations of TC, triglyceride and LDL increased in the 4-VCD + HFD only compared with the DZQE and Control groups suggests that DZQE improves lipid metabolism in perimenopause complicated with hyperlipidaemia in rats. The DZQE groups not only exhibited decreased serum concentrations of TC, triglyceride and LDL in the 4-VCD + HFD group but also showed a reduced concentration of very low-density lipoprotein. This suggests that administration of DZQE could lead to the modulation of a series of biological events that regulate lipid metabolism.

In addition, ALT and AST were significantly increased in the 4-VCD + HFD group but were significantly reduced in the DZQE groups. Severe hepatic steatosis was found in the 4-VCD + HFD group, and DZQE treatment significantly improved hepatic steatosis, suggesting its protective role in regulating lipid metabolism devoid of liver toxicity.

Unravelling the underlying mechanisms through which DZQE confers salutary effects against perimenopause complicated with hyperlipidaemia will not only enhance current knowledge on the biological action of DZQE, but will also lead to the development of new and effective pharmacological agents. AMP-activated protein kinase (AMPK) is a sensor of energy status that regulates energy metabolism including lipid metabolism (Kohjima *et al.* 2008). Therefore, in this study we placed the AMPK pathway as the centre of effort to elucidate the possible mechanism through which DZQE improves ovarian function and regulates lipid metabolism in the rat model of perimenopause complicated with hyperlipidaemia. Our study showed that *AMPK* mRNA and P-AMPK protein levels in the 4-VCD + HFD group decreased significantly compared with the Control group. However, the levels of *AMPK* mRNA and P-AMPK protein in the DZQE administration groups were significantly upregulated compared with the 4-VCD + HFD group. Furthermore, we observed that DZQE treatment resulted in the inhibition of gene expression of *SREBP-1c* as well as its downstream target gene, *FAS*. This corroborates the assertion that DZQE regulates the AMPK pathway and increases the expression of P-AMPK and inhibit *SREBP* expression levels. This demonstrates that DZQE plays an important role in regulating the AMPK pathway to improve lipid metabolism. Acetyl-CoA carboxylase is also an important substrate for AMPK activity. Activation of AMPK inactivates ACC phosphorylation and inhibits malonyl CoA production to reduce fatty acid synthesis (Kudo *et al.* 1996). Acetyl-CoA is responsible for producing malonyl CoA, a carnitine palmitoyl transferase 1 (CPT1) inhibitor (Dobrzyn *et al.* 2004, Hénique *et al.* 2015). AMPK regulates phosphorylation of ACC to promote oxidation of long-chain fatty acyl-CoA esters by activating CPT1α.

Our research confirmed that DZQE increased the phosphorylation of AMPK and ACC in rats fed a high-fat diet and activated AMPK. These results can be interpreted to suggest that DZQE activation of AMPK, downregulation of *ACC* mRNA transcription and inhibition of SREBP could result in reduced fatty acid synthesis. This suggests that administration of DZQE could reduce the synthesis of fatty acids, increase the oxidation of fatty acids and lessen fat deposition in the liver, at least in part through the AMPK signalling pathway.

Danzhi Qing'e actives the AMPK pathway to regulate several intracellular metabolic systems to generate energy. Foremost, the metabolic modifications include the acceleration of lipid catabolism via the suppression of ACC in the liver. For that reason, AMPK protein may be considered to be a pharmacological target for the management of hyperlipidaemia. Danzhi Qing'e can serve to prevent the hyperlipidaemia that underlies the fatty liver disease associated with high fat intake. The liver can regulate the fat uptake and generate energy through the AMPK pathway.

Conclusions

This research confirmed that DZQE could improve ovarian function and lipid metabolism in an animal model of perimenopausal hyperlipidaemia. We also showed that DZQE could correct the reduced production of hormones during ovarian dysfunction in female rats. Furthermore, we contended that the possible mechanism underlying the salutary actions of DZQE involves the AMPK activation pathway. We showed that DZQE poses no liver toxicity as revealed by the organ weights and pathological examination. This study revealed DZQE as a pharmacotherapy that could improve ovarian function and lipid metabolism in perimenopause complicated with hyperlipidaemia rats, at least in part through activation of the AMPK pathway. Therefore, this study has defined a therapeutic approach to improve ovarian function and attenuate lipid accumulation in order to prevent perimenopause-induced ovarian dysfunction and hyperlipidaemia. Safe recommendations for human use, during the treatment of menopausal syndrome, would require further investigation e.g. animal research and/or a clinical trial. A future study to delineate in detail the mechanisms responsible for the salutary effects of DZQE

in the treatment of perimenopausal syndrome is also warranted.

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Additional information

Competing interests

None declared.

Author contributions

Xiaoxue Xing conceived and designed the experiment, analysed the data. Xiaoxue Xing, Patrick Fordjour Asare, Lingyan Wang, Lina Su and Lan Li performed the experiment, drafted the pictures. Xiaoxue Xing, Lina Su, Patrick Fordjour Asare, Lingyan Wang Lan Li, Erwei Liu, Bing Yu, Maojuan Guo, Yan Zhu Xiumei Gao, Guanwei Fan approved the final version of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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