Synergic hypocholesterolaemic effect of *n*-3 PUFA and oestrogen by modulation of hepatic cholesterol metabolism in female rats

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Abstract

n-3 PUFA such as EPA and DHA as well as oestrogen have been reported to decrease blood levels of cholesterol, but their underlying mechanism is unclear. The purpose of this study was to determine the effects of the combination of *n*-3 PUFA supplementation and oestrogen injection on hepatic cholesterol metabolism. Rats were fed a modified AIN-93G diet with 0, 1 or 2 % *n*-3 PUFA (EPA + DHA) relative to the total energy intake for 12 weeks. Rats were surgically ovariectomised at week 8, and, after 1-week recovery, rats were injected with 17β-oestradiol-3-benzoate (E_2) or maize oil for the last 3 weeks. Supplementation with *n*-3 PUFA and E_2 injection significantly increased the ratio of the hepatic expression of phosphorylated AMP activated protein kinase (p-AMPK):AMP activated protein kinase (AMPK) and decreased sterol regulatory element-binding protein-2, 3-hydroxy-3-methylglutaryl coenzyme A reductase and proprotein convertase subtilisin/kexin type 9. Supplementation with *n*-3 PUFA increased hepatic expression of cholesterol 7α-hydroxylase (CYP7A1), sterol 12α-hydroxylase (CYP8B1) and sterol 27-hydroxylase (CYP27A1); however, E_2 injection decreased CYP7A1 and CYP8B1 but not CYP27A1. Additionally, E_2 injection increased hepatic expression of oestrogen receptor-α and β. In conclusion, *n*-3 PUFA supplementation and E_2 injection had synergic hypocholesterolaemic effects by down-regulating hepatic cholesterol synthesis (*n*-3 PUFA and oestrogen) and up-regulating bile acid synthesis (*n*-3 PUFA) in ovariectomised rats.

Key words: Cholesterol metabolism: Oestrogen: *n*-3 PUFA: Ovariectomised rats: Proprotein convertase subtilisin/kexin type 9

Menopause is associated with unfavourable blood-cholesterol changes such as elevated total cholesterol (TC), TAG and LDL-cholesterol and reduced HDL-cholesterol[1]. Dyslipidaemia, especially elevated blood LDL-cholesterol, is a major risk factor for CVD[1]. Post-menopausal women have increased levels of LDL-cholesterol and TC as compared with pre-menopausal women, all of these contribute to an atherogenic lipid profile[1,2]. Post-menopausal women are, therefore, at increased risk for CVD.

Oestrogen injection has been shown to decrease blood levels of LDL-cholesterol by reducing hepatic cholesterol synthesis via down-regulation of sterol regulatory element-binding protein-2 (*SREBP-2*) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMG-CoA reductase*)[2–4]. As a new drug target for lowering LDL-cholesterol, proprotein convertase subtilisin/kexin type 9 (*PCSK9*) is also decreased by oestrogen, suggesting that oestrogen prevents the degradation of the LDL receptor[5,6]. However, oestrogen has also been suggested to induce cholestasis by inhibiting biliary cholesterol secretion and bile acid synthesis via down-regulating expression of cholesterol 7α-hydroxylase (*CYP7A1*)[7,8].

Unlike oestrogen, *n*-3 PUFA, EPA (20 : 5*n*-3) and DHA (22 : 6*n*-3) have been shown to decrease cholesterol by increasing hepatic bile acid synthesis by up-regulation of *CYP7A1*, sterol 12α-hydroxylase (*CYP8B1*) and sterol 27-hydroxylase (*CYP27A1*)[9,10]. In addition to increasing synthesis of bile acids, *n*-3 PUFA decrease blood levels of cholesterol by down-regulating *SREBP-2* and *HMG-CoA reductase* and, thus, decreasing hepatic cholesterol synthesis[11,12]. However, the effect of *n*-3 PUFA on hepatic expression of PCSK9 has not been studied.

Bravo *et al.*[13] reported that supplementation with *n*-3 PUFA reduced plasma levels of LDL-cholesterol in male rats injected with oestrogen by increasing the number of LDL receptors, but hepatic cholesterol synthesis was not studied. Although the effect of *n*-3 PUFA on cholesterol concentration is not entirely clear, a recent meta-analysis reported an increase in LDL-cholesterol by *n*-3 PUFA[14]. The LDL-cholesterol-increasing effects of *n*-3 PUFA have been shown in patients with hypertriacylglycerolaemia[15] and hamsters consuming high-fat diets[16] but not in healthy volunteers[17]. Therefore, the purpose of the present study was to determine the effects of the

combination of *n*-3 PUFA supplementation and oestrogen injection on hepatic cholesterol synthesis and breakdown, including the effects on PCSK9 expression, in ovariectomised rats (OVX) fed a low-fat diet.

Methods

Animals

A total of forty-eight female Wistar rats aged 3 weeks (Jungang Lab Animal Inc.) were used for the present study. All experimental protocols were adhered to the institutional guidelines for the care and management of laboratory animals, and approved by the Institutional Animal Care and Use Committee of Hanyang University (registration no. HY-IACUC-12-076).

Study design

Rats were individually housed under conditions of constant temperature (22 (SEM 1)°C) and humidity (47 (SEM 1)%) and a 12 h light–12 h dark cycle. Food pellets and fresh tap water were available *ad libitum* and food intake was measured daily. The body weight of the rats was determined weekly for 12 weeks.

After 1 week of acclimation, rats were randomly divided into three isoenergetic diet groups (*n* 16 each) and fed a modified American Institute of Nutrition (AIN) 93G diet for 12 weeks. Soyabean oil in the AIN-93G diet was substituted with same amount of grape seed oil (CJ CheilJedang) and/or fish oil (Cenovis Health Company) in order to achieve an EPA + DHA content of 0, 1 or 2% of the total energy. Grape seed oil contained very low levels of α-linolenic acid (18 : 3*n*-3), EPA and DHA. Per kg of diet, there was 42·94 g total fat (10% of energy) and 0, 1 or 2% *n*-3 PUFA (EPA + DHA) equal to 0, 8·09 or 16·21 g of fish oil, respectively. The fatty acid composition of the diets is shown in Table 1.

At week 8, rats were surgically ovariectomised under anaesthesia using a combination of tiletamine/zolazepam

(Zoletil, 25 ml/kg; Virbac S.A.) and xylazine (Rumpun, 10 ml/kg; Bayer Korea). Starting 1 week after ovariectomisation, rats (*n* 8/group) were randomly assigned to groups injected with either 17*β*-oestradiol-3-benzoate (E$_2$) or the vehicle only (maize oil; Sigma-Aldrich) for the last 3 weeks of the study (week 10–12); the six groups were: 0% *n*-3 PUFA diet with maize oil injection (0% *n*3), 0% *n*-3 PUFA diet with E$_2$ injection (0% *n*3 + E$_2$), 1% *n*-3 PUFA diet with maize oil injection (1% *n*3), 1% *n*-3 PUFA diet with E$_2$ injection (1% *n*3 + E$_2$), 2% *n*-3 PUFA diet with maize oil injection (2% *n*3) and 2% *n*-3 PUFA diet with E$_2$ injection (2% *n*3 + E$_2$). At 12 week of the experiment, rats were fasted overnight and euthanised with an intraperitoneal injection of Zoletil (25 ml/kg) and Rumpun (10 ml/kg). Blood was collected, and serum was obtained after centrifugation. Organs were collected, washed with saline solution and weighed. Serum and tissue samples were stored at –80°C until analysis.

Determination of serum and liver lipid

Hepatic lipid was extracted according to the method of Folch *et al.*[18]. Hepatic and serum concentrations of TC, TAG and serum HDL-cholesterol were measured using commercial kits (Asan Pharmaceutical) according to manufacturer's instructions. The concentration of LDL-cholesterol was calculated using the formula of Friedewald *et al.*[19].

GC analysis

Serum fatty acid composition was measured as previously reported[20]. Briefly, 12·5 µl of serum was methylated with 500 µl of boron trifluoride methanol-benzene (Sigma-Aldrich) for 45 min at 100°C. Fatty acid methyl esters were extracted with 500 µl of hexane and analysed by GC (Shimadzu 2010) using a SP2560 capillary column (100 m × 0·25 mm i.d., 0·2 µm film thickness, Supelco). Fatty acids were identified by comparing retention times with standards (GLC-727; Nu-Check Prep). Every batch was analysed with a quality control sample, and the CV was <5%.

Western blot analysis

For analysis, extracts were prepared by homogenising the liver tissue in lysis buffer (0·25 M-sucrose, 20 mM-*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid, 2 mM-dithiothreitol, 1 mM-phenylmethanesulfonylfluoride, 0·5 mM-EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM-Na$_3$VO$_4$, pH 7·5) plus a phosphatase inhibitor cocktail tablet (Roche Life Science). After centrifugation at 20 000 ***g*** for 1 h at 4°C (Eppendorf 5417R; Eppendorf), the supernatant was collected as a cytosolic fraction. To obtain the nuclear fraction, the pellets were re-suspended in lysis buffer with 1% (v/v) Triton X-100, and centrifuged at 20 000 ***g*** for 30 min at 4°C. The supernatant was used for analysis of nuclear proteins. For protein quantification, the Bradford method was applied using bovine serum albumin (Bio-Rad) as a standard. Individual lysates (30–80 µg of protein) were separated on an 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes (0·45 µm, Merck Millipore, Billerica, MA, USA).

Antibodies used in this study included the following: AMP activated protein kinase (AMPK) and phosphorylated AMPK

Table 1. Fatty acid composition of the diets

Fatty acids (%)	0% *n*3	1% *n*3	2% *n*3
14 : 0	0·09	0·16	0·23
16 : 0	6·87	6·70	6·53
16 : 1*n*-7	0·11	0·15	0·18
18 : 0	3·63	4·20	4·78
18 : 1*n*-9	14·72	14·12	13·53
18 : 2*n*-6	73·15	59·58	45·95
18 : 3*n*-3	0·44	0·47	0·49
20 : 4*n*-6	0·01	0·34	0·67
20 : 5*n*-3	0·02	6·17	12·34
22 : 4*n*-6	0·02	0·35	0·68
22 : 5*n*-6	0·01	0·12	0·24
22 : 5*n*-3	0·02	0·86	1·71
22 : 6*n*-3	0·01	4·86	9·74
SFA	10·61	11·11	11·62
MUFA	15·08	14·82	14·56
n-3 PUFA	0·48	12·36	24·28
n-6 PUFA	73·24	60·97	48·65
n-3:*n*-6 ratio	0·01	0·20	0·50

0, 1 and 2% *n*3, % *n*-3 PUFA relative to the total energy intake in the diet.

(p-AMPK) from Cell Signaling Technology; HMG-CoA reductase, CYP7A1 and CYP8B1 from Santa Cruz Biotechnology; and SREBP-2, PCSK9, CYP27A1, oestrogen receptor-α (ER-α) and oestrogen receptor-β (ER-β) from Abcam.

After blocking with 5 % skimmed milk or 5 % bovine serum albumin in tris-buffered saline with 0·1 % tween for 1 h at room temperature, membranes were incubated overnight at 4°C with the following primary antibodies: AMPK (1:500), p-AMPK (1:1000), HMG-CoA reductase (1:100), CYP7A1 (1:250), CYP8B1 (1:250), SREBP-2 (1:1000), PCSK9 (1:1000), CYP27A1 (1:1000), ER-α (1:500) and ER-β (1:500). Secondary antibody incubation was performed using horseradish peroxidase-conjugated anti-mouse IgG (1:5000; Enzo Life Science), anti-goat (1:10 000; Santa Cruz Biotechnology) or anti-rabbit (1:10 000; Cell Signaling Technology) for 1 h at room temperature. The immunoreactive proteins were developed using the enhanced chemiluminescence kit (GE Healthcare Life Sciences) and quantified using the Chemidoc MP Imaging System (Bio-Rad). The relative amounts of the proteins were calculated by normalising to the quantity of β-actin (1:1000; BD Transduction Laboratories).

Statistical analyses

All data are expressed as mean values with their standard errors. Statistical differences were calculated with a two-way ANOVA. All the statistical analyses were performed using SPSS for Windows, version 18.0 (SPSS Inc.). *P* values <0·05 were considered statistically significant.

Results

Food intake, body weight and organ weight

Supplementation with *n*-3 PUFA had no significant effect on dietary intake, body weight or organ weight (Table 2; see online Supplementary Table S1). E$_2$ injection, however, significantly decreased dietary intake, final body weight and visceral fat weight, whereas it increased liver, uterus and kidney weight regardless of *n*-3 PUFA supplementation. E$_2$ injection had no significant effect on the weight of the liver or kidneys.

Serum and hepatic lipid

Supplementation with *n*-3 PUFA significantly and dose-dependently decreased the serum levels of TC, LDL-cholesterol and TAG, and the hepatic levels of TC and TAG (Table 3; see online Supplementary Table S2). Similarly, E$_2$ injection significantly decreased the serum levels of LDL-cholesterol and TAG and the hepatic levels of TC and TAG. However, serum levels of TC were not significantly affected by E$_2$ injection. Serum levels of HDL-cholesterol were significantly increased by E$_2$ injection but not by *n*-3 PUFA supplementation. There was a significant synergistic effect of *n*-3 PUFA supplementation and E$_2$ injection on serum LDL-cholesterol and hepatic TC levels.

Serum fatty acid composition

Supplementation with *n*-3 PUFA significantly increased serum levels of total *n*-3 PUFA, 20 : 5*n*-3, 22 : 5*n*-3 and 22 : 6*n*-3, while

Table 2. Dietary intake, body weight and organ weight of rats
(Mean values with their standard errors)

	0 % *n*3		1 % *n*3		2 % *n*3		0 % *n*3 + E$_2$		1 % *n*3 + E$_2$		2 % *n*3 + E$_2$	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Dietary intake (g/d)	14·96	0·11	15·22	0·16	15·13	0·15	14·34*	0·10	14·19*	0·10	14·35*	0·03
Initial body weight (g)	93·31	1·74	93·56	1·93	96·13	2·13	93·44	2·02	94·13	2·23	94·38	2·04
Final body weight (g)	294·13	3·23	299·00	2·76	301·00	3·96	260·31*	2·22	263·00*	3·20	263·50*	2·84
Liver (mg/g body weight)	21·82	1·00	21·60	2·21	21·47	1·56	24·44*	1·06	24·27*	0·55	24·14*	0·77
Uterus (mg/g body weight)	0·30	0·02	0·31	0·02	0·30	0·03	1·49*	0·14	1·54*	0·17	1·56*	0·07
Visceral fat (mg/g body weight)	13·78	1·38	14·33	2·03	14·74	2·22	10·80*	1·79	9·82*	1·08	9·39*	1·38
Kidney (mg/g body weight)	5·71	0·19	5·63	0·35	5·72	0·25	6·35*	0·23	6·38*	0·14	6·44*	0·41

0, 1 and 2 % *n*3, 0, 1 and 2 % *n*-3 PUFA diet with maize oil injection; 0, 1 and 2 % *n*3 + E$_2$, 0, 1 and 2 % *n*-3 PUFA diet + 17β-oestradiol-3-benzoate (E$_2$), 0, 1 and 2 % *n*-3 PUFA diet with E$_2$ injection.
* Values are significantly different between maize oil and (E$_2$) injection for diets containing the same amount of *n*-3 PUFA (*P* <0·05).

Table 3. Serum and hepatic lipid profile
(Mean values with their standard errors)

	0 % *n3*		1 % *n3*		2 % *n3*		0 % *n3* + E₂		1 % *n3* + E₂		2 % *n3* + E₂	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Serum												
TC (mmol/l)	2·69	0·12	2·47†	0·05	2·18†‡	0·07	2·69	0·07	2·44†	0·06	2·08†‡	0·08
HDL-cholesterol (mmol/l)	0·71	0·02	0·71	0·04	0·71	0·02	0·93*	0·05	0·92*	0·05	0·96*	0·02
LDL-cholesterol (mmol/l)	1·67	0·10	1·44†	0·06	1·19†‡	0·06	1·47*	0·07	1·25*†	0·02	0·97*†‡	0·13
TAG (mmol/l)	0·66	0·04	0·58†	0·03	0·48†‡	0·02	0·58*	0·02	0·48*†	0·01	0·41*†‡	0·02
Liver												
TC (mg/g liver)	2·55	0·06	2·35†	0·07	2·18†‡	0·05	2·12*	0·03	1·97*†	0·03	1·81*†‡	0·04
TAG (mg/g liver)	64·84	4·31	54·77†	3·90	46·11†‡	3·83	27·92*	1·78	18·78*†	1·43	13·23*†	0·74

0, 1 and 2 % *n3*, 0, 1 and 2 % *n*-3 PUFA diet with maize oil injection; 0, 1 and 2 % *n3* + 17β-oestradiol-3-benzoated (E₂), 0, 1 and 2 % *n*-3 PUFA diet with E₂ injection; TC, total cholesterol.
* Values are significantly different between maize oil and E₂ injection for diets containing the same amount of *n*-3 PUFA (*P* < 0·05).
† Values are significantly different among 0, 1 and 2 % *n3* within the maize oil and E₂ injected groups (*P* < 0·05).
‡ Values are significantly different between 1 and 2 % *n3* within the maize oil and E₂ injected groups (*P* < 0·05).

decreasing the serum levels of total *n*-6 PUFA 20 : 4*n*-6 in a dose-dependent manner (see online Supplementary Table S3). E₂ injection decreased the serum levels of 14 : 0, 16 : 0, 16 : 1*n*-7 and 18 : 1*n*-9 but increased the concentration of 18 : 0. In addition, E₂ injection significantly increased the serum levels of 22 : 5*n*-3 and 22 : 6*n*-3 in rats fed *n*-3 PUFA. There was a significant interaction between *n*-3 PUFA supplementation and E₂ injection on the serum levels of 22 : 6*n*-3, total *n*-3 PUFA, 22 : 5*n*-6 and total *n*-6 PUFA.

Hepatic protein expression related with cholesterol metabolism

Supplementation with *n*-3 PUFA and E₂ injection significantly decreased the expression of HMG-CoA reductase, SREBP-2 and PCSK9, whereas it increased the ratio of the expression of p-AMPK:AMPK (Fig. 1). Hepatic expressions of p-AMPK and AMPK were also increased both by *n*-3 PUFA supplementation and E₂ injection (see online Supplementary Fig. S1).
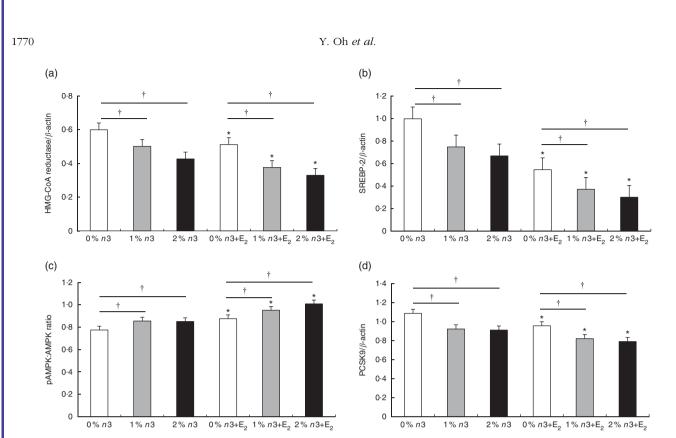
Supplementation with *n*-3 PUFA significantly increased the expression of CYP7A1, CYP8B1 and CYP27A1 (Fig. 2). However, E₂ injection significantly decreased the expression of CYP8B1 but not of CYP27A1 (Fig. 2). In addition, E₂ injection significantly increased the hepatic expression of ER-*α* and ER-*β*, but *n*-3 PUFA did not (Fig. 3).

Discussion

This was the first report to suggest that E₂ injection and *n*-3 PUFA supplementation have a synergic hypocholesterolaemic effect through inhibited hepatic cholesterol synthesis and increased breakdown of hepatic cholesterol in OVX. Consistent with our results, Bravo *et al*.[13] reported that E₂ injection and *n*-3 PUFA supplementation decreased LDL-cholesterol by increasing the amount of LDL receptors in male rats. The LDL receptor binds with *PCSK9*, which enhances LDL receptor degradation and results in increased plasma levels of LDL-cholesterol[21,22]. The present study showed that E₂ injection and *n*-3 PUFA supplementation synergistically reduced hepatic expression of *PCSK9*, which inhibited LDL receptor degradation and decreased LDL-cholesterol. Previously, E₂ injection decreased *PCSK9* expression in male rats[5], but the effect of *n*-3 PUFA had not been investigated.

PCSK9 has been shown to be regulated by SREBP-2, which communicates with hepatic proteins involved in cholesterol synthesis such as p-AMPK, AMPK and HMG-CoA reductase[23]. Previous preclinical studies have suggested that E₂ injection[4,24] or *n*-3 PUFA supplementation[11,25] reduce cholesterol synthesis by increasing *AMPK* phosphorylation, reducing expression of *HMG-CoA reductase* and reducing expression of *SREBP-2*. Consistently, the present study shows that the reduced hepatic cholesterol synthesis was due to an up-regulation of p-AMPK and AMPK, an increase in the p-AMPK:AMPK ratio, and a down-regulation of HMG-CoA reductase and SREBP-2. However, reports on the effects of *n*-3 PUFA on LDL-cholesterol have been contradictory; some have proposed that rapid TAG clearance by lipoprotein lipase can promote the conversion of VLDL to LDL and thus increase circulating LDL-cholesterol[26].

Fig. 1. The effect of *n*-3 PUFA supplementation and 17β-oestradiol-3-benzoated (E$_2$) injection on expression of (a) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), (b) sterol regulatory element-binding protein-2 (SREBP-2), (c) phosphorylated AMP activated protein kinase (p-AMPK):AMP activated protein kinase (AMPK) ratio and (d) proprotein convertase subtilisin/kexin type 9 (PCSK9) in the liver of ovariectomised rats. Values are means (*n* 8 rats/group), with their standard errors represented by vertical bars. * Mean values are significantly different between maize oil and E$_2$ injection for diets containing the same amount of *n*-3 PUFA (*P* < 0·05). † Mean values are significantly different among 0, 1 and 2 % *n*3 within the maize oil and E$_2$ injected groups. 0, 1 and 2 % *n*3, 0, 1 and 2 % *n*-3 PUFA diets with maize oil injection; 0, 1 and 2 % *n*3 + E$_2$, 0, 1 and 2 % *n*-3 PUFA diets with E$_2$ injection (*P* < 0·05).

The LDL-cholesterol-increasing effects of *n*-3 PUFA have been reported in hypertriacylglycerolaemia[15], but not in normo-triacylglycerolaemia[17], as in the present study.

Another mechanism that has been shown to reduce blood levels of cholesterol is increased hepatic cholesterol breakdown. Bile acids promote hepatic cholesterol removal, and thus an increase in bile acid synthesis results in a reduction of blood cholesterol[10]. Previous studies reported that *n*-3 PUFA supplementation increased hepatic expression of *CYP7A1*, *CYP8B1* and *CYP27A1*[9,10] in mice, suggesting enhanced bile acid synthesis and reduced hepatic levels of cholesterol. On the other hand, oestrogen has been shown to decrease hepatic synthesis of bile acids through a reduction of *CYP7A1* and *CYP8B1* but not *CYP27A1*, in rats and mice[8,27]. Similarly, the present study showed that E$_2$ injection suppressed hepatic expression of *CYP7A1* and *CYP8B1* but not *CYP27A1*. The effect of oestrogen could be related to the expression of endoplasmic reticulum-localised enzymes such as *CYP7A1* but not the mitochondria-localised enzyme *CYP27A1*[28]. *CYP7A1* regulates *CYP8B1*, which mediates bile acid synthesis through cholic acid synthesis[29], and the suppression of *CYP8B1* (e.g. by E$_2$) causes a reduction of *CYP7A1* expression. Although E$_2$ injection did not increase the hepatic synthesis of bile acids in this study, it suppressed hepatic cholesterol synthesis that, in turn, decreased hepatic and blood levels of cholesterol. Previous studies have suggested that oestrogen induces cholestasis through reducing hepatic *CYP7A1*
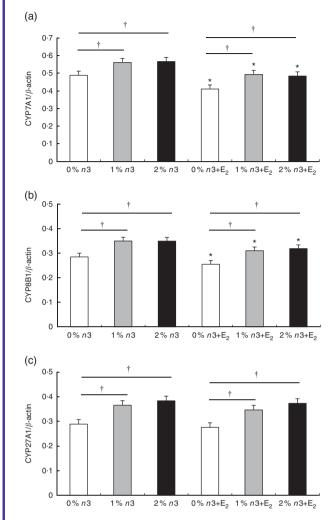
and biliary cholesterol secretion[8,27]. Therefore, it is worth noting that the combination of *n*-3 PUFA and E$_2$ in the present study could have decreased blood and hepatic levels of cholesterol by decreasing hepatic cholesterol synthesis and increasing hepatic synthesis of bile acids.

In the liver, E$_2$ binds to oestrogen receptors (ER), specifically ER-α and ER-β subtypes, to modulate cholesterol metabolism. The binding of E$_2$ to ER results in the recruitment of co-activators and the displacement of co-repressors at DNA binding sites and thereby the modulation of gene and protein expression[30] including LDL receptors in liver. Thus, oestrogen can up-regulate the expression of the LDL receptor through binding to ER and increasing the clearance of blood LDL-cholesterol[31]. In the present study, E$_2$ injection increased the hepatic expression of *ER-α* and *ER-β*, but *n*-3 PUFA did not. This result is consistent with a previous study in which oestrogen increased the amount of hepatic ER in OVX rats[32]. However, the effect of *n*-3 PUFA on ER has not yet been reported.

In the present study, E$_2$ injection decreased serum levels of 14 : 0, 16 : 0, 16 : 1*n*-7 and 18 : 1*n*-9 but increased 18 : 0. The elongase 6 enzyme elongates 16 : 0 to 18 : 0; this was demonstrated in elongase 6 knockout mice who exhibited greater concentrations of 16 : 0 but less 18 : 0 compared with wild-type mice[33]. In addition, elongase 6 expression was higher in females than males and was increased in oestrogen-treated HepG2 cells[34]. Consistently, the present study showed

Fig. 2. The effect of *n*-3 PUFA supplementation and 17β-oestradiol-3-benzoated (E₂) injection on the expression of (a) cholesterol 7 α-hydroxylase (CYP7A1), (b) sterol 12 α-hydroxylase (CYP8B1) and (c) sterol 27-hydroxylase (CYP27A1) in the liver of ovariectomised rats. Values are means (*n* 8 rats/group), with their standard errors represented by vertical bars. * Mean values are significantly different between maize oil and E₂ injection for diets containing the same amount of *n*-3 PUFA (*P* < 0·05). † Mean values are significantly different among 0, 1 and 2 % *n*3 within the maize oil and E₂ injected groups. 0, 1 and 2 % *n*3, 0, 1 and 2 % *n*-3 PUFA diets with maize oil injection; 0, 1 and 2 % *n*3 + E₂, 0, 1 and 2 % *n*-3 PUFA diets with E₂ injection (*P* < 0·05).

that E₂ injection increased 18 : 0 concentrations through activation of elongase 6. Additionally, oestrogen has been shown to inhibit stearoyl-CoA desaturase-1[35], the rate-limiting enzyme in the synthesis of MUFA. In the present study, concentrations of MUFA 16 : 1*n*-7 and 18 : 1*n*-9 were depressed in rats injected with E₂. Furthermore, E₂ injection and *n*-3 PUFA supplementation increased serum levels of 22 : 5*n*-3 and 22 : 6*n*-3 synergistically. E₂ injection increased serum levels of 22 : 5*n*-6 in the 0 % *n*3 diet, as observed previously in plasma in rats[36]. Previous studies reported that dietary *n*-3 PUFA[37,38] and oestrogen[36] increased blood levels of *n*-3 PUFA and decreased levels of *n*-6 PUFA. Oestrogen increases Δ6-desaturase, which converts 18 : 3*n*-3 to 20 : 5*n*-3 and 22 : 6*n*-3[36].
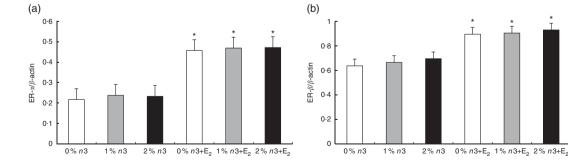
This study had a few limitations. Although rats are the most commonly used model for lipoprotein research, lipoprotein metabolism differs between rats and human. Differences are the efficient mechanisms for clearance of remnant-removal pathways and absence of cholesteryl ester transfer reaction[39]. There was no sham surgery group in the present study, but previous studies compared cholesterol metabolism between OVX and sham rats[3,40]. In addition, the present study did not measure bile acid concentrations of faecal or biliary secretion.

In conclusion, *n*-3 PUFA supplementation and E₂ injection significantly reduced hepatic and blood levels of cholesterol by inhibiting hepatic cholesterol synthesis through increasing AMPK phosphorylation, decreasing PCSK9, SREBP-2 and HMG-CoA reductase expression, and enhancing hepatic cholesterol breakdown through CYP7A1, CYP8B1 and CYP27A1. Further research is needed to determine the effects of *n*-3 PUFA supplementation and hormone replacement therapy on cholesterol metabolism in post-menopausal women.

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Fig. 3. The effect of *n*-3 PUFA supplementation and 17β-oestradiol-3-benzoated (E₂) injection on expression of (a) oestrogen receptor-*α* (ER-*α*) and (b) oestrogen receptor-*β* (ER-*β*) in the liver of ovariectomised rats. Values are means (*n* 8 rats/group), with their standard errors represented by vertical bars. * Mean values are significantly different between maize oil and E₂ injection for diets containing the same amount of *n*-3 PUFA (*P* < 0·05). 0, 1 and 2 % *n*3, 0, 1 and 2 % *n*-3 PUFA diets with maize oil injection; 0, 1 and 2 % *n*3 + E₂, 0, 1 and 2 % *n*-3 PUFA diets with E₂ injection (*P* < 0·05).

manuscript and has primary responsibility for the final content. All authors read and approved the final manuscript.

None of the authors has any conflicts of interest to declare.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114515003517

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