Synergistic attenuation of ovariectomy-induced bone loss by combined use of fish oil and 17β-oestradiol

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Abstract

Oestrogen and n-3 PUFA, especially EPA and DHA, have been reported to have beneficial effects on bone loss. Thus, the purpose of the present study was to investigate the synergistic bone-protective mechanism of combined treatments of EPA + DHA supplementation and oestrogen injection in ovariectomised rats. Rats were fed a modified American Institute of Nutrition-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 a 1-week recovery period rats were injected with either 17β-oestradiol-3-benzoate (E_2) or maize oil for the last 3 weeks. Combined use of n-3 PUFA and E_2 synergistically increased femoral cortical bone volume, bone mineral content and the bone expression of runt-related transcription factor 2 (RUNX2), but decreased the bone expression of IL-1β. Both n-3 PUFA and E_2 decreased the bone expressions of IL-7, TNF-α and PPAR-γ, and increased the bone expression of oestrogen receptor-α. n-3 PUFA in the presence of E_2 and E_2 alone significantly decreased the bone expressions of IL-1β and IL-6 and increased the bone expression of RUNX2. E_2 significantly decreased the serum levels of bone turnover markers and the bone expression of receptor activator of NF-κB ligand, but decreased the bone expression of osteoprotegerin. The combined use of n-3 PUFA and E_2 exerted synergistic bone-protective efficacy through up-regulation of RUNX2, an essential transcription factor for bone formation, as well as the suppression of bone-resorbing cytokine IL-1β.

Key words: **17β-Oestradiol-3-benzoate: n-3 PUFA: Bone loss: Runt-related transcription factor 2: IL-1β**

Ageing is associated with bone loss, and postmenopausal women are at a particularly high risk of osteoporosis, as oestrogen deficiency is the other primary factor in increasing bone resorption[1]. Oestrogen deficiency has been known to up-regulate osteoclast formation, which is mainly induced by the increased production of bone-resorbing inflammatory cytokines such as IL-1β, IL-6, IL-7 and TNF-α[2]. In ovariectomised (OVX) rats, 17β-oestradiol-3-benzoate (E_2) treatment maintained bone volume (BV) by regulating both bone resorption and bone formation[3]. In *in vitro* studies, E_2 exerted anti-resorptive efficacy by regulating the receptor activator of NF-κB ligand (RANKL), an important determinant of osteoclast differentiation, and osteoprotegerin (OPG), which binds to and inactivates RANKL[4–6]. In addition, E_2 increased the activity of runt-related transcription factor 2 (RUNX2)[7], an essential factor for differentiation of osteoblasts.

In addition to oestrogen, the n-3 PUFA EPA (20 : 5n-3) and DHA (22 : 6n-3) are known to reduce osteoclastic bone resorption by decreasing the production of eicosanoids and inflammatory cytokines[8]. In Fat-1 OVX mice, a transgenic model synthesising n-3 PUFA from n-6 PUFA, osteoblastogenesis was promoted by the inhibited expression of PPAR-γ and the enhanced expression of RUNX2 in bone marrow, which resulted in increased bone mass compared with wild-type OVX mice[9].

There have been several studies investigating the combined effects of n-3 PUFA and E_2 treatment on bone mass and architecture[10–12]. A combination of E_2 treatment and dietary supplementation of γ-linolenic acid + EPA[10], DHA[11] or flaxseed oil rich in α-linolenic acid[12] was more effective in preserving OVX-induced bone loss compared with either treatment alone. However, there have been no studies examining the underlying mechanisms by which n-3 PUFA alone or n-3 PUFA with E_2 enhanced bone-protective efficacy. Therefore, the purpose of the present study was to investigate the synergistic bone-protective mechanism of the combined treatment of EPA + DHA supplementation and E_2 injection in OVX rats.

Methods

Animals and diet

This animal protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-12-076). In total, 3-week-old female Wistar rats (Jung Ang Lab. Animal Inc.) were kept in ventilated cages in an air-conditioned room under controlled temperature ($22 \pm 1°C$) and humidity (40–50 %) with a 12-h light–12 h dark cycle. Rats had free access to food pellets and tap water. Rats were weighed once a week, and food intake was measured every day. A modified American Institute of Nutrition-93G diet with 10 % fat relative to total energy intake was used. The diet contained 42·94 g/kg of fish oil (Cenovis Health Company) and/or grape seed oil (CJ Cheilje-dang). Grape seed oil was used instead of soyabean oil, which contained $18:3n$-3. The diets contained 0 %, 1 % or 2 % of EPA + DHA relative to the total energy and contained 0, 8·09 and 16·21 g of fish oil, 0·2, 5·3 and 10·4 g of n-3 PUFA, and 31·4, 26·2 and 20·9 g of n-6 PUFA per kg diet, respectively. The fatty acid

composition of the diet is shown in Table 1, as previously described in detail[13]. In brief, the 0 %, 1 % and 2 % EPA + DHA diets contained 0·44, 0·47 and 0·49 % of $18:3n$-3, 0·02, 6·17 and 12·34 of $20:5n$-3, 0·02, 0·86 and 1·71 % of $22:5n$-3, and 0·01, 4·86 and 9·74 % of $22:6n$-3, respectively.

Experimental design

After a 1-week acclimatisation, 60 rats (4 weeks old) were randomly divided into one of three isoenergetic experimental diets (0 %, 1 % and 2 % EPA + DHA) for 12 weeks (n 20 per diet). At 8 weeks, rats (11 weeks old) were surgically OVX under anaesthesia. After a 1-week recovery period from ovariectomy, rats (n 10 per group) were randomly assigned to E_2- or maize oil-injected groups (Sigma-Aldrich): 0 % EPA + DHA diet with maize oil injection (0 % n3), 0 % EPA + DHA diet with E_2 injection (0 % n3 + E_2), 1 % EPA + DHA diet with maize oil injection (1 % n3), 1 % EPA + DHA diet with E_2 injection (1 % n3 + E_2), 2 % EPA + DHA diet with maize oil injection (2 % n3) and 2 % EPA + DHA diet with E_2 injection (2 % n3 + E_2). The schematic diagram for group allocation is presented in Fig. 1. To mimic the rat oestrous cycle, 1 ml of maize oil with or without 10 µg of E_2 was subcutaneously injected every 4 days during the last 3 weeks of the experiment, with the final administration performed 24 h before killing. Rats (15 weeks old) were fasted overnight and were anaesthetised with zoletil (25 mg/kg) and rompun (10 mg/kg). Blood samples were collected in serum-separating tubes and EDTA blood tubes, and serum and plasma were obtained after centrifugation. Tissues were collected, rinsed with saline and weighed at the time of killing. Femura and tibiae were dissected, and the length and weight of the bones were measured. Blood and tissue samples were snap-frozen in liquid N_2 and stored at –80°C for further analysis.
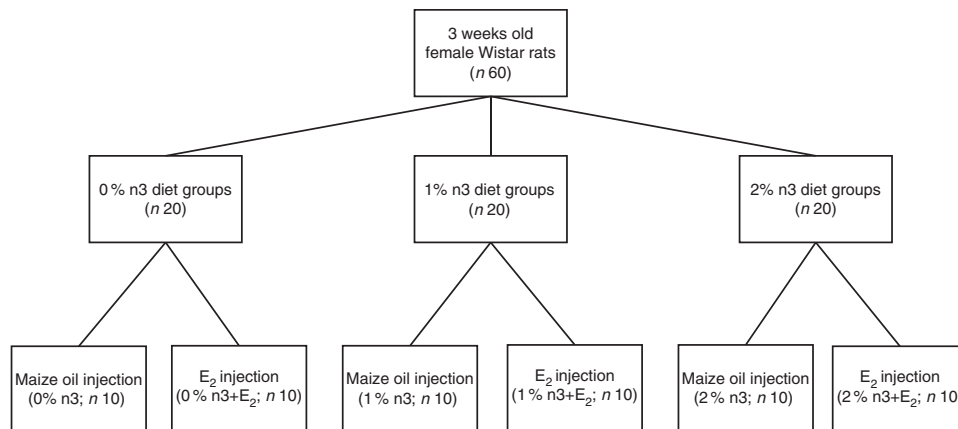
Measurement of blood levels of 17β-oestradiol-3-benzoate and eicosanoids

Plasma level of E_2 (Cayman Chemical Company) and serum levels of PG E_2 (Cayman Chemical Company) and leukotriene

Table 1. Fatty acid composition of the diets

Fatty acids (%)	0 % n3	1 % n3	2 % n3
14:0	0·09	0·16	0·23
16:0	6·87	6·70	6·53
16:1n-7	0·11	0·15	0·18
18:0	3·63	4·20	4·78
18:1n-9	14·72	14·12	13·53
18:2n-6	73·15	59·58	45·95
18:3n-3	0·44	0·47	0·49
20:4n-6	0·01	0·34	0·67
20:5n-3	0·02	6·17	12·34
22:4n-6	0·02	0·35	0·68
22:5n-6	0·01	0·12	0·24
22:5n-3	0·02	0·86	1·71
22:6n-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 % n3, % n-3 PUFA relative to the total energy intake in the diet.

Fig. 1. Schematic diagram of group allocation. After a 1-week acclimatisation period, sixty rats were randomly divided into one of three isoenergetic experimental diets (0 %, 1 % and 2 % EPA + DHA) for 12 weeks (n 20 per group). At 8 weeks, rats were surgically ovariectomised, and after a 1-week recovery period from ovariectomy, rats were randomly assigned to 17β-oestradiol-3-benzoate (E_2)- or maize oil-injected groups (n 10 per group). 0 %, 1 % and 2 % n3, 0 %, 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E_2, 0 %, 1 % and 2 % EPA + DHA diet with E_2 injection.

B$_4$ (LTB$_4$; R&D Systems) were measured using ELISA according to the manufacturer's instructions. All measurements were quantified relative to standards in a spectrophotometer (Multiscan GO; Thermo Scientific).

Determination of blood levels of calcium, phosphate and bone turnover markers

Serum levels of calcium (Ca) and phosphate (P) were measured using colorimetric methods (BioAssay Systems) and analysed using a microplate reader (iMark Microplate Reader; Bio-Rad). Serum levels of bone formation markers such as osteocalcin (OC; Nordic Bioscience) and bone-specific alkaline phosphatase (BSALP; MyBioSource) and a bone resorption marker, C-terminal cross-linked telopeptides of type I collagen (CTX; Nordic Bioscience), were measured using ELISA according to the manufacturer's instructions. The results were quantified in a spectrophotometer.

GC

Total lipids were extracted from powdered femur (*n* 10 per group; 300 mg) by homogenisation in 5-ml chloroform–methanol–distilled water (2:2:1, v/v), and phospholipids were separated with TLC (Silica gel G; Analtech) using hexane–diethyl ether–acetic acid (40:10:1, v/v). Phospholipids were methylated by adding boron trifluoride methanol–benzene (B1252; Sigma-Aldrich), and were heated at 100°C for 10 min. Fatty acid methyl esters were analysed using GC (Shimadzu 2010AF; Shimadzu Scientific Instruments). An external standard (GLC-727; Nu-Check Prep) was used for identification of fatty acids, and the CV was 2·0 %.

Micro-computed tomography

Left femora (*n* 5 per group) were fixed in 4 % paraformaldehyde after killing. A high-resolution micro-computed tomography system (InspeXio SMX-90CT, Shimadzu, Kyoto, Japan) was used to scan the proximal region of the femora. The micro-computed tomography (μCT) machine is equipped with a 90-kV scanning voltage, 10-W power, 109-μA current and 9-μm scan thickness. The femora were placed on a holder between the X-ray source and the flat panel detector (inspeXio SMX--90CT; Shimadzu). The scanning angular rotation was 360°, and the angular increment was 1·6°. After scanning the data, reconstruction of three-dimensional data sets with a voxel size of 9 μm was conducted. Both femoral trabecular and cortical bones were analysed using the commercial software provided with the equipment (TRI3D-BON Analysis software; RATOC).

Western blot analysis

Frozen femora (*n* 10 per group) were ground into a fine powder with a mortar and pestle under liquid N$_2$. Bone powder was homogenised in an ice-cold radioimmunoprecipitation buffer containing complete EDTA-free protease and PhosSTOP inhibitor cocktails (Roche Diagnostics GmbH). The homogenates were centrifuged at 10 000 ***g*** for 15 min at 4°C, and the protein concentration of the supernatant was determined using a bicinchoninic assay (Pierce Biotechnology). Proteins (30 μg) were separated on 8–10 % SDS-PAGE, transferred to polyvinylidene fluoride membranes and blocked for 1 h at room temperature with 5 % skimmed milk or 5 % bovine serum albumin in Tris-buffered saline containing Tween 20 (TBST). The membrane was then incubated with anti-PPAR-γ (1:400), RUNX2 (1:500), OPG (1:1000), RANKL (1:2000), TNF-α (1:1000), IL-6 (1:1000), IL-1β (1:1000), IL-7 (1:400) and oestrogen receptor-α (ER-α; 1:500) or ER-β (1:1000) with 5 % skimmed milk in TBST overnight at 4°C. All antibodies were purchased from Abcam, except for anti-IL-7. After rinsing in TBST three times for 5 min each, membranes were incubated with horseradish-peroxidase-conjugated secondary antibody, which was either anti-rabbit IgG (Cell signaling, New England Biolabs) or anti-mouse IgG (Enzo Life Science) with 5 % skimmed milk in TBST for 1 h at room temperature. Immuno-reactive bands were visualised on the UV setting using the ChemiDoc MP Imaging System (Bio-Rad) to estimate total protein per lane, and β-actin was used for normalisation.

Statistical analysis

Values are expressed as means with their standard errors , and differences were considered significant at $P < 0.05$. Data were analysed using a two-way ANOVA with factors of *n*-3 PUFA diet and E$_2$ injection, followed by Duncan's *post hoc* test. Analyses were conducted using SPSS for Windows, version 18.0 (SPSS Inc.).

Results

Dietary intake, body weight and organ weights

There were no significant differences in initial body weight, weights of the liver or kidneys, and weight and length of the tibia and femur (Table 2). Regardless of *n*-3 PUFA supplementation, E$_2$-injected rats consumed less food and had a lower final body weight but heavier uterus weight relative to maize oil-injected rats.

Blood concentrations of 17β-oestradiol-3-benzoate, eicosanoids, calcium, phosphate and bone turnover markers

E$_2$ injection significantly increased the plasma concentration of E$_2$ and the serum level of Ca but decreased the serum levels of OC, BSALP and CTX regardless of *n*-3 PUFA supplementation (Table 3). Both *n*-3 PUFA supplementation and E$_2$ injection decreased the serum level of LTB$_4$, and an additive inhibitory effect was observed in the combined treatment of *n*-3 PUFA and E$_2$. There were no effects of *n*-3 PUFA or E$_2$ on the blood levels of PG E$_2$ and P.

Fatty acid composition of femur phospholipids

n-3 PUFA supplementation significantly increased the bone phospholipid levels of 18 : 2*n*-6 and *n*-3 PUFA such as 20 : 5*n*-3, 22 : 5*n*-3 and 22 : 6*n*-3, whereas supplementation decreased the levels of *n*-6 PUFA such as 20 : 4*n*-6, 22 : 4*n*-6 and 22 : 5*n*-6 in a dose-dependent manner (Table 4). E$_2$ injection significantly

Table 2. Dietary intake and body and organ weights
(Mean values with their standard errors; n 10 per group)

| | Treatments | | | | | | | | | | | | P-value | | |
| | 0 % n3 | | 1 % n3 | | 2 % n3 | | 0 % n3 + E_2 | | 1 % n3 + E_2 | | 2 % n3 + E_2 | | | | |
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	E_2	n3	$E_2 \times$ n3
Dietary intake (g/d)	15·02	0·20	15·24	0·23	15·44	0·14	14·28*	0·18	14·14*	0·14	14·38*	0·20	<0·001	0·319	0·559
Initial body weight (g)	96·10	1·66	97·60	3·43	95·10	3·76	94·95	3·13	94·10	2·99	90·90	2·68	0·236	0·589	0·870
Final body weight (g)	296·15	6·24	298·30	5·58	308·20	7·22	258·85*	4·52	265·10*	4·44	265·60*	4·15	<0·001	0·236	0·692
Liver weight (g)	6·21	0·18	6·62	0·21	6·73	0·26	6·46	0·23	6·45	0·14	6·67	0·17	0·960	0·208	0·558
Kidney weight (g)	1·66	0·05	1·67	0·05	1·67	0·04	1·69	0·04	1·73	0·03	1·75	0·04	0·091	0·630	0·800
Uterus weight (mg)	81·70	4·36	106·40	16·01	91·70	4·40	402·40*	26·64	435·70*	25·02	402·50*	12·25	<0·001	0·208	0·866
Tibia weight (mg)	702·90	15·80	738·50	20·89	755·40	22·64	706·90	21·70	737·00	16·60	729·20	35·74	0·750	0·269	0·724
Femur weight (mg)	910·40	35·48	924·70	35·77	957·70	24·82	900·90	17·49	979·40	23·42	981·70	28·79	0·324	0·075	0·532
Tibia length (cm)	3·92	0·04	4·01	0·03	3·99	0·05	3·96	0·08	3·98	0·04	4·00	0·05	0·813	0·310	0·732
Femur length (cm)	3·65	0·05	3·65	0·02	3·70	0·01	3·60	0·07	3·69	0·04	3·63	0·06	0·457	0·537	0·476

0 %, 1 % and 2 % n3, 0 % 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E_2, 0 %, 1 % and 2 % EPA + DHA diet with 17β-oestradiol-3-benzoate injection.
* Mean values was significantly different between maize oil and E_2 injection within the diets containing the same amount of n-3 PUFA at $P < 0.05$.

Table 3. Blood levels of 17β-oestradiol-3-benzoate (E_2), eicosanoids, calcium, phosphate and bone turnover marker levels
(Mean values with their standard errors; n 10 per group)

| | Treatments | | | | | | | | | | | | P-value | | |
| | 0 % n3 | | 1 % n3 | | 2 % n3 | | 0 % n3 + E_2 | | 1 % n3 + E_2 | | 2 % n3 + E_2 | | | | |
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	E_2	n3	$E_2 \times$ n3
E_2 (pmol/l)	31·35	6·61	48·17	11·67	67·59	16·59	146·16*	13·22	169·95*	14·43	206·11*	60·50	<0·001	0·221	0·906
PG E_2 (nmol/l)	3·50	0·24	3·10	0·17	2·43	0·64	2·74	0·25	3·00	0·28	2·81	0·25	0·567	0·291	0·253
LTB$_4$ (nmol/l)	2·09	0·16	1·58**	0·14	1·59**	0·16	1·53*	0·13	1·18*	0·11	1·13*,**	0·09	<0·001	<0·001	0·838
Ca (mmol/l)	271·47	1·75	273·72	2·99	275·71	3·74	308·90*	3·99	306·65*	2·99	301·16*	2·00	<0·001	0·814	0·153
P (mmol/l)	1·80	0·11	1·79	0·13	1·65	0·14	1·85	0·12	1·64	0·04	1·73	0·07	0·941	0·403	0·529
OC (nmol/l)	78·34	2·73	79·59	2·39	79·67	4·12	54·84*	2·06	48·23*	1·64	47·24*	2·40	<0·001	0·453	0·198
BSALP (U/l)	4·73	0·88	4·59	0·51	4·44	0·60	2·42*	0·38	1·94*	0·21	1·56*	0·16	<0·001	0·539	0·859
CTX (pmol/l)	172·66	4·73	162·36	7·97	163·42	10·16	108·05*	4·44	100·36*	3·24	113·27*	5·22	<0·001	0·346	0·488

0 %, 1 % and 2 % n3, 0 %, 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E_2; 0 %, 1 % and 2 % EPA + DHA diet with 17β-oestradiol-3-benzoate injection; LTB$_4$, leukotriene B$_4$; OC, osteocalcin; BSALP, bone-specific alkaline phosphatase; CTX, C-terminal cross-linked telopeptides of type I collagen.
* Values are significantly different between maize oil and E_2 injection within the diets containing the same amount of EPA + DHA at $P < 0.05$.
** Values are significantly different among 0 %, 1 % and 2 % n3 within the maize oil- or E_2-injected group at $P < 0.05$.

Table 4. Fatty acid compositions of femur phospholipids
(Mean values with their standard errors; *n* 10 per group)

	Treatments												P-value		
	0 % n3		1 % n3		2 % n3		0 % n3 + E₂		1 % n3 + E₂		2 % n3 + E₂				
Fatty acids (%)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	E₂	n3	E₂ × n3
14 : 0	0·53	0·03	0·50	0·02	0·59	0·02	0·52	0·04	0·53	0·04	0·54	0·04	0·744	0·315	0·485
16 : 0	29·75	0·34	30·91	0·91	30·74	0·42	29·38	0·52	30·58	0·86	29·91	0·50	0·325	0·177	0·911
18 : 0	16·61	0·25	16·14	0·24	16·57	0·17	17·78*	0·10	17·86*	0·21	17·77*	0·15	<0·001	0·543	0·291
18 : 1*n*-9	12·30	0·13	12·50	0·20	12·57	0·16	11·15*	0·15	11·24*	0·10	11·56*	0·13	<0·001	0·077	0·690
18 :·2*n*-6	8·74	0·24	10·38**	0·20	10·45**	0·28	8·48	0·34	10·58**	0·30	10·97**	0·32	0·511	<0·001	0·389
18 : 3*n*-3	0·11	0·01	0·12	0·00	0·12	0·01	0·12	0·01	0·12	0·00	0·12	0·01	0·231	0·536	0·884
20 : 4*n*-6	19·94	0·47	15·44**	0·54	12·39** ***	0·33	20·63	0·30	15·04**	0·44	12·90** ***	0·27	0·423	<0·001	0·359
20 : 5*n*-3	0·11	0·02	1·00**	0·05	2·15** ***	0·10	0·09	0·00	0·89**	0·02	2·00** ***	0·11	0·069	<0·001	0·656
22 : 4*n*-6	3·40	0·07	1·70**	0·07	1·08** ***	0·05	3·47	0·05	1·68**	0·03	0·96** ***	0·07	0·575	<0·001	0·240
22 : 5*n*-6	3·54	0·19	0·28**	0·02	0·21**	0·01	3·67	0·13	0·23**	0·02	0·19**	0·02	0·778	<0·001	0·606
22 : 5*n*-3	0·34	0·06	2·22**	0·04	3·12** ***	0·09	0·26	0·02	2·28**	0·05	3·01** ***	0·08	0·424	<0··001	0·369
22 : 6*n*-3	1·53	0·15	5·54**	0·33	6·29**	0·36	1·56	0·13	6·02**	0·35	6·74**	0·57	0·265	<0·001	0·774
n-3 PUFA	2·09	0·20	8·92**	0·34	11·79** ***	0·42	2·02	0·14	9·29**	0·39	11·89** ***	0·70	0·678	<0·001	0·863
n-6 PUFA	36·58	0·52	28·90**	0·64	25·21** ***	0·4	37·79	0·61	28·57**	0·59	26·04** ***	0·30	0·396	<0·001	0·517
SFA	47·07	0·54	47·76	0·89	48·13	0·48	47·86	0·56	49·20	0·86	48·45	0·64	0·130	0·290	0·712
MUFA	13·59	0·15	13·83	0·26	13·93	0·17	12·27*	0·18	12·32*	0·11	12·77*	0·20	<0·001	0·080	0·645

0 %, 1 % and 2 % n3, 0 %, 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E₂, 0 %, 1 % and 2 % EPA + DHA diet with 17β-oestradiol-3-benzoate injection.
* Values are significantly different between maize oil and E₂ injection within the diets containing the same amount of EPA + DHA at $P < 0.05$.
** Values are significantly different among 0 %, 1 % and 2 % n3 within maize oil- or E₂-injected groups at $P < 0.05$.
*** Values are significantly different between 1 % and 2 % n3 within maize oil- or E₂-injected groups at $P < 0.05$.

increased the bone phospholipid level of 18 : 0 but decreased the levels of 18 : 1*n*-9 and MUFA.

The expressions of cytokines, osteoprotegerin, receptor activator of NF-κB ligand, PPAR-γ and runt-related transcription factor 2 in bone

E₂ injection significantly decreased the bone expression of the inflammatory cytokines IL-1β, IL-6, IL-7 and TNF-α regardless of *n*-3 PUFA supplementation (Fig. 2). *n*-3 PUFA supplementation significantly decreased the bone expressions of IL-7 and TNF-α regardless of E₂ injection, but decreased the bone expressions of IL-1β and IL-6 only in rats injected with E₂. In addition, there were significant interactions between *n*-3 PUFA and E₂ in the bone expression of IL-1β ($P = 0.021$), indicating the synergistic reduction of IL-1β expression by the combination of *n*-3 PUFA and E₂.

E₂ injection, but not *n*-3 PUFA supplementation, significantly increased the bone expression of OPG and decreased the bone expression of RANKL (Fig. 3). Both *n*-3 PUFA supplementation and E₂ injection decreased the bone expression of PPAR-γ. The bone expression of RUNX2 was increased by E₂ injection only in rats supplemented with *n*-3 PUFA and by *n*-3 PUFA supplementation only in rats injected with E₂. Interactions between *n*-3 PUFA and E₂ were also seen in the bone expression of RUNX2 ($P = 0.041$), suggesting a synergistic increase in RUNX2 expression by the combination of *n*-3 PUFA and E₂.

Micro-computed tomography analysis (bone microarchitecture)

Representative μCT images of the analysed region of the femur are shown in Fig. 4. There was no significant difference in femoral trabecular bone mineral density (BMD) after either *n*-3 PUFA supplementation or E₂ injection. Femoral trabecular BV/ tissue volume (TV) and bone mineral content (BMC)/TV were significantly increased by E₂ injection regardless of *n*-3 PUFA supplementation, but were increased by *n*-3 PUFA supplementation only in rats with E₂ injection. There was a marginally significant interaction between *n*-3 PUFA supplementation and E₂ injection on the femoral trabecular BV/TV ($P = 0.056$).

There was no significant difference in femoral cortical BMD or BV caused by either *n*-3 PUFA supplementation or E₂ injection. However, femoral cortical BMC was increased by *n*-3 PUFA supplementation only in rats with E₂ injection. There was a significant interaction between *n*-3 PUFA supplementation and E₂ injection on femoral cortical BV ($P = 0.008$) and BMC ($P = 0.034$), suggesting synergistic increases in cortical BV and BMC by the combination of *n*-3 PUFA and E₂.

The expressions of oestrogen receptor-α and oestrogen receptor-β in bone

E₂ injection and *n*-3 PUFA supplementation significantly increased the bone expression of ER-α (Fig. 5). However, there was no significant effect of E₂ injection or *n*-3 PUFA supplementation on the bone expression of ER-β.

Discussion

This was the first study to demonstrate that the synergistic bone-protective efficacy of *n*-3 PUFA and E₂ was induced by suppression of bone-resorbing cytokine IL-1β, followed by the enhanced bone expression of RUNX2, the major transcription factor in osteoblast differentiation.

A combination of E₂ treatment and dietary supplementation of *n*-3 PUFA, including γ-linolenic acid + EPA[10], DHA[11] or flaxseed oil rich in α-linolenic acid[12], has been reported to be
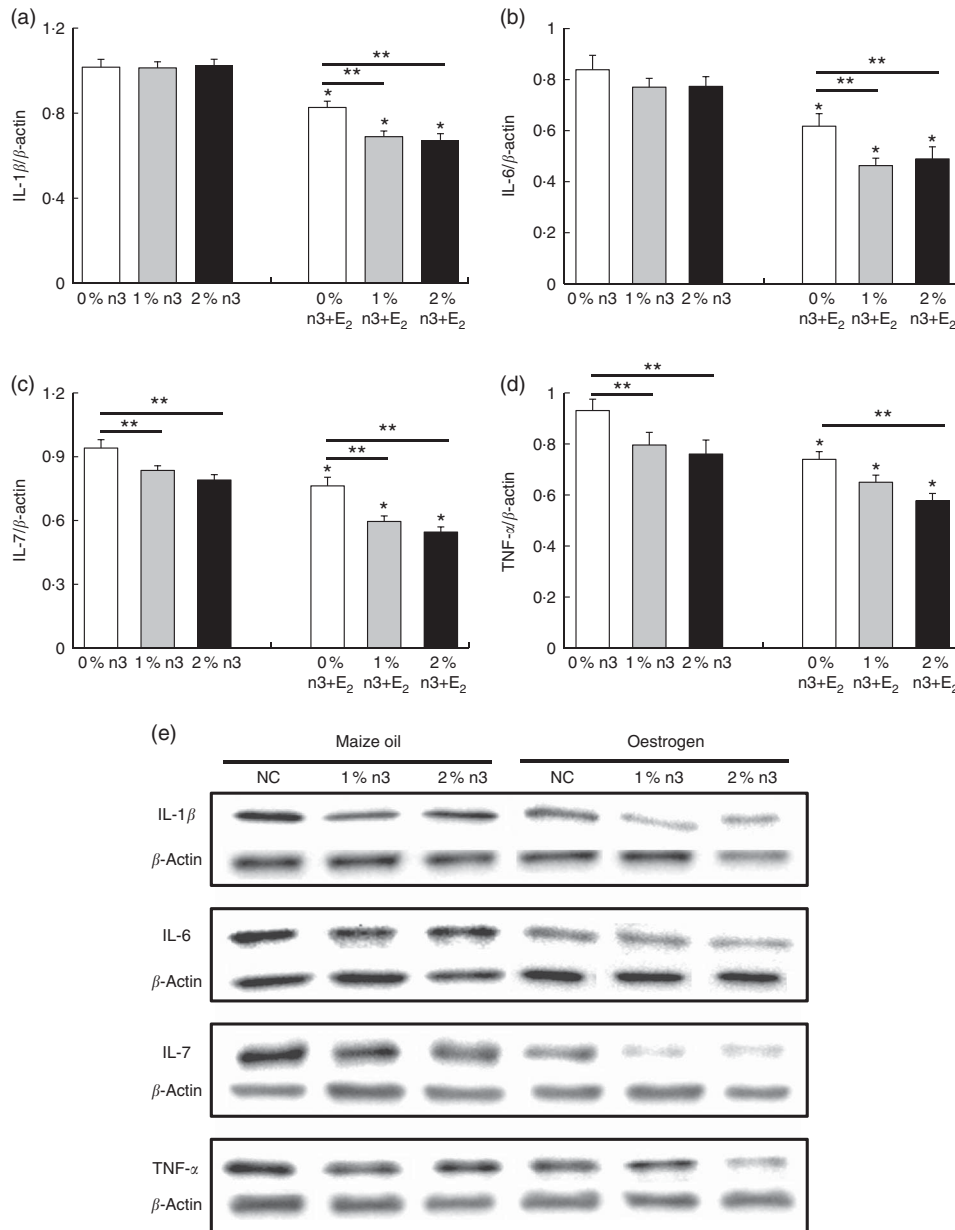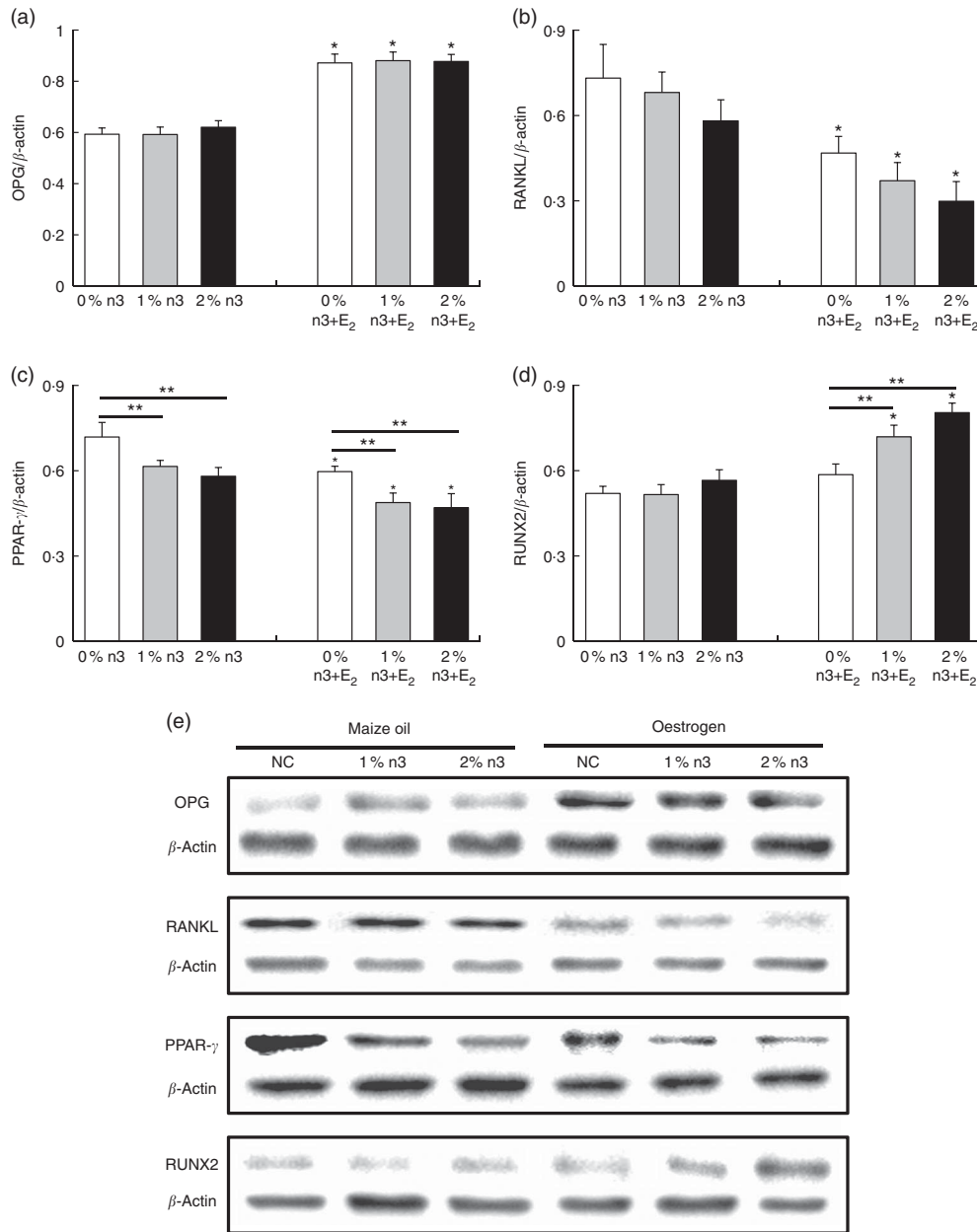
Fig. 2. Effects of *n*-3 PUFA supplementation and 17β-oestradiol-3-benzoate (E₂) on the bone expressions of (a) IL-1β, (b) IL-6, (c) IL-7, (d) TNF-α and (e) representative images of Western blotting. Values are means (*n* 10 per group), with their standard errors are represented by vertical bars. 0 %, 1 % and 2 % n3, 0 %, 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E₂, 0 %, 1 % and 2 % EPA + DHA diet with E₂ injection. * Values are significantly different between maize oil and E₂ injection within the diets containing the same amount of EPA + DHA at *P* < 0·05. ** Values are significantly different among 0 %, 1 % and 2 % n3 within maize oil- or E₂-injected groups, at *P* < 0·05. NC, 0 % n3.

more effective in preserving OVX-induced bone loss than if used separately. Consistent with previous studies, the present study found a synergistic combined effect of *n*-3 PUFA (EPA + DHA) and E₂ on femoral cortical BMC and BV, as well as on bone expressions of RUNX2 and IL-1β. RUNX2 is well known for its vital role in osteoblastogenesis[14], whereas IL-1β is one of the major cytokines stimulating osteoclast differentiation and accelerating bone resorption[15]. Ding *et al.*[16] reported that RUNX2 expression is inhibited by IL-1β in human mesenchymal stem cells (MSC), suggesting an interaction between IL-1β and RUNX2. A synergistic decrease in IL-1β expression through the

combined treatment of *n*-3 PUFA and E₂ might result in a subsequent up-regulation of RUNX2, which can contribute to the maintenance of bone mass and BV observed in the present study. On the other hand, RUNX2 has been reported to be associated with the bone-specific signalling of E₂ through an ER-α dependent pathway[7]. As the present study showed that the combined use of *n*-3 PUFA and E₂ enhanced the ER-α expression, RUNX2 expression might be modulated through an ER-α-dependent pathway.

Consistent with previous studies[3,17], the present study found that E₂ treatment reduces OVX-induced bone loss and serum
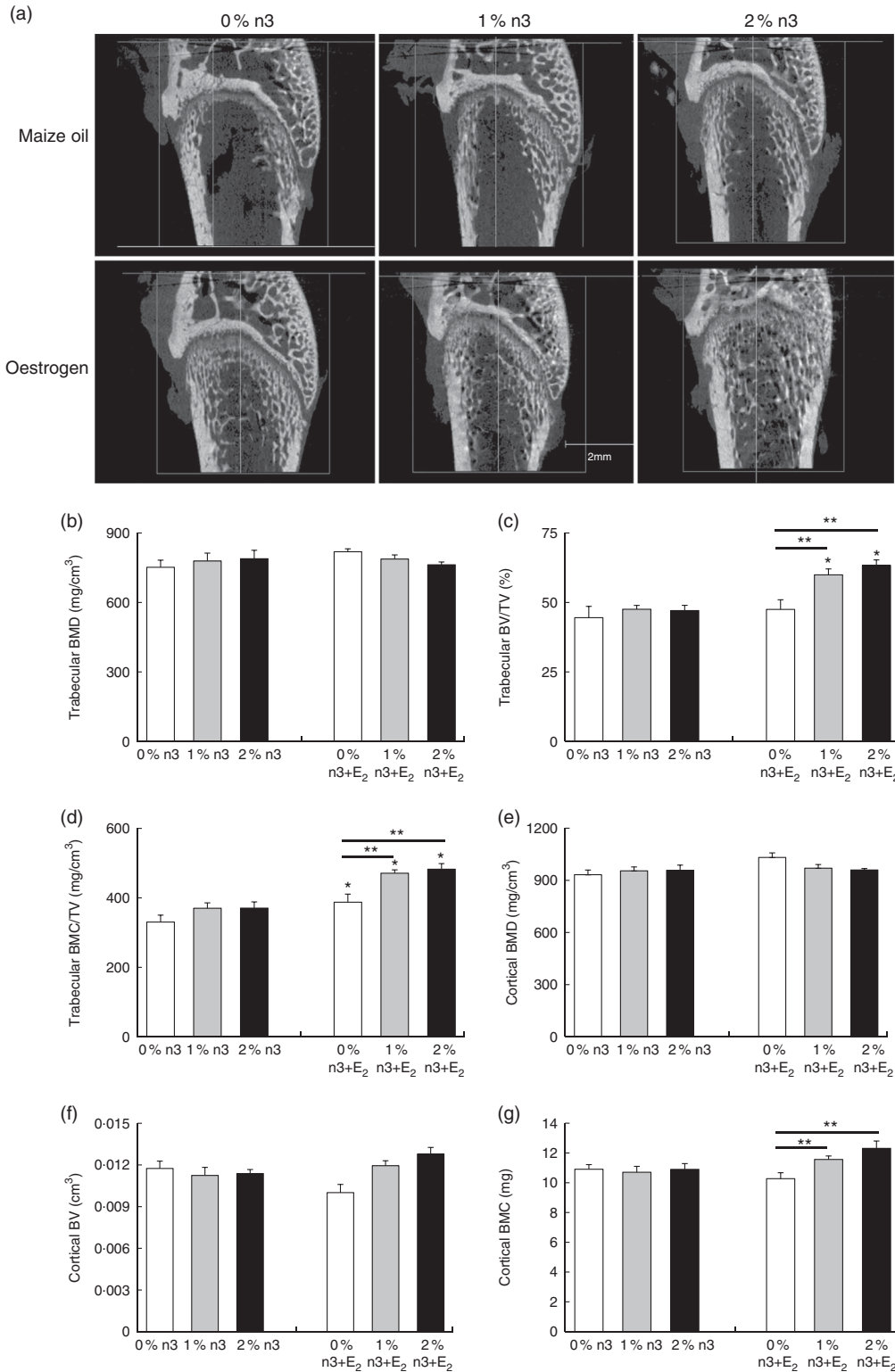
Fig. 3. Effects of *n*-3 PUFA supplementation and 17β-oestradiol-3-benzoate (E$_2$) on the bone expressions of (a) osteoprotegerin (OPG), (b) receptor activator of NF-κB ligand (RANKL), (c) PPAR-γ, (d) runt-related transcription factor 2 (RUNX2) and (e) representative images of Western blotting. Values are means (*n* 10 per group), with their standard errors are represented by vertical bars. 0 %, 1 % and 2 % n3, 0 %, 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E$_2$, 0 %, 1 % and 2 % EPA + DHA diet with E$_2$ injection. * Values are significantly different between maize oil and E$_2$ injection within the diets containing the same amount of EPA + DHA at $P < 0.05$. ** Values are significantly different among 0 %, 1 % and 2 % n3 within maize oil- or E$_2$-injected groups, at $P < 0.05$. NC, 0 % n3.

levels of bone turnover markers such as OC, BSALP and CTX. It is well known that oestrogen deficiency increases bone turnover and induces rapid bone loss[18]. In addition, our study showed a significant increase in serum levels of Ca in rats injected with E$_2$, which is in line with previous results reporting that E$_2$ treatment increased intestinal Ca absorption[19].

One of the mechanisms by which E$_2$ regulates bone metabolism could be related to the suppression of osteoclast differentiation[2]. Previous studies have shown that E$_2$ treatment reduced osteoclastogenesis through stimulation of OPG secretion but decreased RANKL and cytokine expressions[5,6,20],

consistent with the present study. In addition, E$_2$ promotes osteoblastogenesis by enhancing RUNX2 activity[7] or by increasing the number of cells expressing RUNX2[21], which inhibits bone marrow adipogenesis by reducing PPAR-γ expression[22]. The present study also found that the bone expression of RUNX2 significantly increased but that of PPAR-γ decreased with E$_2$ treatment.

Oestrogen can act on the skeleton through two receptors, ER-α and ER-β, both of which are present in bone, although ER-α appears to mediate most actions of oestrogen in bone[23]. In the present study, E$_2$ increased the bone expression of ER-α

Fig. 4. Micro-computed tomography analysis of the proximal femur from control and *n*-3 PUFA-supplemented and/or 17β-oestradiol-3-benzoate (E$_2$)-injected rats. (a) Representative three-dimensional images of the proximal femur. Effects of *n*-3 PUFA and E$_2$ on femoral trabecular (b) bone mineral density (BMD), (c) bone volume (BV)/tissue volume (TV) and (d) bone mass content (BMC)/TV. Effects of *n*-3 PUFA and E$_2$ on femoral cortical (e) BMD, (f) BV and (g) BMC. Values are means (*n* 10 per group), with their standard errors are represented by vertical bars. 0 %, 1 % and 2 % n3, 0 %, 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E$_2$, 0 %, 1 % and 2 % EPA + DHA diet with E$_2$ injection. * Values are significantly different between maize oil and E$_2$ injection within the diets containing the same amount of EPA + DHA at *P* < 0.05. ** Values are significantly different among 0 %, 1 %, and 2 % n3 within maize oil- or E$_2$-injected groups, at *P* < 0.05.
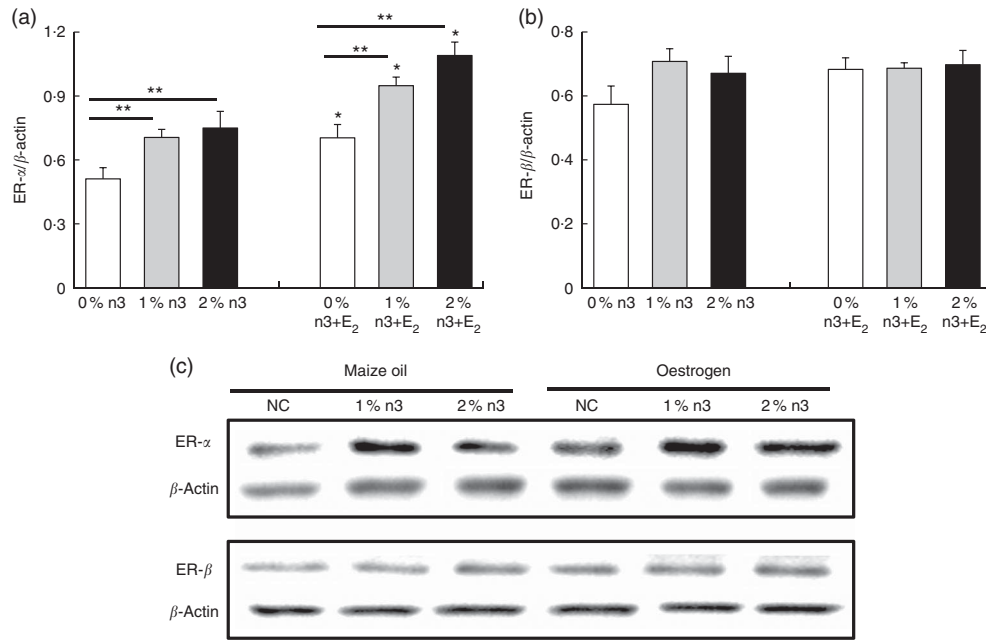
Fig. 5. Effects of *n*-3 PUFA supplementation and 17β-oestradiol-3-benzoate (E$_2$) on the bone expressions of (a) oestrogen receptor-α (ER-α), (b) ER-β and (c) representative images of Western blotting. Values are mean (*n* 10 per group), with their standard errors are represented by vertical bars. 0 %, 1 % and 2 % n3, 0 %, 1 % and 2 % EPA + DHA diet with maize oil injection; 0 %, 1 % and 2 % n3 + E$_2$, 0 %, 1 % and 2 % EPA + DHA diet with E$_2$ injection. * Values are significantly different between maize oil and E$_2$ injection within the diets containing the same amount of EPA + DHA at $P < 0.05$. ** Values are significantly different among 0 %, 1 % and 2 % n3 within maize oil- or E$_2$-injected groups, at $P < 0.05$.

but not of ER-β, consistent with a previous finding showing increased ER-α mRNA in human bone marrow cells treated with E$_2$[24]. Activation of ER-α by E$_2$ suppressed osteoclastogenesis[23] and stimulated OPG expression in osteoblasts[4].

Previous studies have shown that *n*-3 PUFA supplementation reduced OVX-induced bone loss in rats[11,25,26], whereas the present study found that *n*-3 PUFA prevented femoral BV and BMC depletion in OVX rats with E$_2$ but not in those without E$_2$. Although rats were fed *n*-3 PUFA for 12 weeks in the present study, the lack of bone-protective efficacy of *n*-3 PUFA alone might be due to the relatively short feeding period after OVX compared with previous studies (4 weeks *v.* 12–18 weeks)[11,25,26]. As bone loss occurred rapidly after OVX[1], the feeding period of *n*-3 PUFA after OVX should be longer than 12 weeks. In addition, the present study and another study[27] used fish oil containing more EPA than DHA, which failed to prevent OVX-induced bone loss, whereas fish oil with high DHA levels demonstrated a bone-protective effect[27,28].

In the present study, *n*-3 PUFA supplementation significantly decreased the serum level of LTB$_4$ and the bone expressions of inflammatory cytokines, which are strong promoters of bone resorption and osteoclastogenesis[29,30]. Rahman *et al.*[31] reported that increased *n*-3 PUFA levels in bone marrow phospholipids in Fat-1 OVX mice were accompanied by reduced production of inflammatory cytokines in bone marrow cells. However, there was no significant effect of *n*-3 PUFA on the bone expression of RANKL or its decoy receptor OPG, which are also important determinants of osteoclastic differentiation and activation[5]. Previous *in vitro* and *in vivo* studies examining the effects of *n*-3 PUFA on the RANKL/OPG pathway

reported mixed results[32–34]. Sacco *et al.*[35] reported that short-term (2 weeks) periods of *n*-3 PUFA (flaxseed oil) supplementation resulted in no changes in the serum level of RANKL or in the OPG:RANKL ratio in OVX rats, but that E$_2$ treatment did result in such changes. Therefore, the lack of regulatory effects of *n*-3 PUFA on the RANKL/OPG pathway might compensate for the effects of reduced inflammatory cytokines on osteoclastic bone loss.

Another mechanism involved in the regulation of bone metabolism by *n*-3 PUFA is the alteration of osteoblast differentiation[36]. Bone formation is mediated by the differentiation of MSC into adipocytes or osteoblasts, which are regulated by the key transcription factors PPAR-γ and RUNX2, respectively[9]. In the present study, *n*-3 PUFA suppressed PPAR-γ expression and complementarily increased the expression of RUNX2 by reducing bone loss in the presence of E$_2$. It has been reported that factors related to MSC differentiation may affect the activity of other regulators, indicating the presence of cross-talk between different signalling pathways[37]. Thus, simultaneous regulation of PPAR-γ and RUNX2 by *n*-3 PUFA in the presence of E$_2$ may additively contribute to osteoblastogenesis and preserve bone loss.

In line with previous studies[26,36], the present study showed that *n*-3 PUFA supplementation increased bone phospholipid levels of long-chain *n*-3 PUFA dose-dependently. In addition, the present study reported that *n*-3 PUFA supplementation decreased bone phospholipid levels of *n*-6 PUFA, except 18 : 2*n*-6. The increased 18 : 2*n*-6 level was because of the fact that the *n*-3 PUFA diet used in the present study contained 18 : 2*n*-6, and long-chain *n*-6 PUFA were decreased because of

the competition of EPA and DHA in membrane incorporation[38]. The decreased bone phospholipid levels of *n*-6 PUFA, especially 20 : 4*n*-6, the substrate for PG E$_2$ synthesis, could partly contribute to the anti-inflammatory effect[8]. In addition, the present study showed that E$_2$ injection increased the bone phospholipid levels of 18 : 0 but decreased the levels of 18 : 1*n*-9, which might be due to the regulatory effects of E$_2$ on stearoyl-CoA desaturase-1 and elongase 6 enzymes[39].

There are several limitations to the present study. There were no sham controls, which made it impossible to analyse OVX-induced bone loss. However, using OVX rats as the animal model for postmenopausal osteoporosis is well-established. In addition, the present study measured bone loss only in the femur, which might be a better site for reflecting osteoporotic bone loss[40], rather than other sites. Although we injected E$_2$ every 4 d to mimic the rat's oestrous cycle, our finding could be applicable to the clinical setting that *n*-3 PUFA might have a bone-protective effect in postmenopausal women, especially who receive hormone-replacement therapy.

Conclusions

This is the first study to reveal that *n*-3 PUFA and E$_2$ exert synergistic bone-protective efficacy through the suppression of bone-resorbing cytokine IL-1β followed by up-regulation of RUNX2, an essential transcription factor of bone formation. The present study suggested a new evidence for the interaction between IL-1β and RUNX2 on bone-protective effect by the combination of *n*-3 PUFA and E$_2$. However, further clinical studies are needed to investigate whether the bone-protective efficacy of *n*-3 PUFA is greater in premenopausal or postmenopausal women receiving hormone therapy than in postmenopausal women not receiving hormone therapy.

Acknowledgements

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