

Effects of Long-Term Administration of N-3 Polyunsaturated Fatty Acids (PUFA) and Selective Estrogen Receptor Modulator (SERM) Derivatives in Ovariectomized (OVX) Mice

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Abstract We studied the beneficial effects of dietary consumption of n-3 polyunsaturated fatty acids (PUFA) and two selective estrogen receptor modulator (SERM) derivatives (SERM-I and SERM-II) and their combined effect on serum lipids, skin dermis and adipose layers, bone marrow adipogenesis, and cytokine secretion in mice. Two different ovariectomized (OVX) models were studied: treatment began immediately post-OVX in one and 3 months post-OVX in the other. Our results showed that n-3 PUFA and both SERMs decreased triglyceride levels in the serum, and that SERMs also decreased serum cholesterol levels while n-3 PUFA had no similar effect. SERMs had no effect on IL-6, IL-1 beta, or IL-10 levels, but they decreased ex vivo tumor necrosis factor (TNF- α). N-3 PUFA decreased secretion of non-induced IL-6 and TNF- α from cultured BMC and IL-1 beta levels in vivo (i.e., in bone marrow plasma), but its main effect was a significant elevation in the secretion of IL-10, a known anti-inflammatory cytokine. OVX-induced B-lymphopoiesis was not affected by LY-139481 (SERM-I) while LY-353381 (SERM-II) exhibited an estrogen-antagonistic effect in sham and OVX mice and elevated the amount of B-cells in bone marrow. Fish oil consumption prevented the elevation in B-lymphopoiesis caused by OVX, but had no curative effect on established augmented B-lymphopoiesis. This activity could be mediated via the elevation of IL-10 which was shown to suppress B-lymphopoiesis. Both SERMs and n-3 PUFA inhibited the increase in adipose tissue thickness caused by OVX in mice. Our results showed that n-3 PUFA, could prevent some of the deleterious outcomes of estrogen deficiency that were not affected by SERMs. We observed no significant beneficial effects of the combined administration of SERM-I, SERM-II, and PUFA on the studied parameters. The exact mechanism by which polyunsaturated fatty acids exert their activities is still not clear, but peroxisome proliferator-activated receptors (PPARs) might be involved in processes which are modulated by n-3 PUFA. *J. Cell. Biochem.* 90: 347–360, 2003. © 2003 Wiley-Liss, Inc.

Key words: omega-3 fatty acids; cytokines; bone-marrow; skin adipocytes; serum lipids; B-lymphopoiesis

Estrogen deficiency caused by ovariectomy (OVX), disease or menopause produces a variety of systemic manifestations. It is associated with atrophy of the uterus [Ashby et al., 1997], bone loss [Peng et al., 1999; Hogan et al., 2000], elevated serum lipids [Walsh et al., 1998; Stark et al., 2000], increased risk for cardiovascular

disease [Stark et al., 2000], loss of thickness and elasticity of skin [Sauerbronn et al., 2000], and decline in immunological properties [Clemett and Spencer, 2000]. Elevation of proinflammatory cytokine levels is another of its consequences. The published results on changes in the levels of cytokines following estrogen withdrawal, however, are controversial; while numerous studies have demonstrated increased production of hematopoietic growth factors (M-CSF) and proinflammatory cytokines, including interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-10 (IL-10) [Cheleuitte et al., 1998; Editorial, 1998; Rodan and Martin, 2000], many others failed to show any change in the levels of

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cytokines post-OVX [Rogers and Eastell, 1998; Van Bezooijen et al., 1998].

Hormone replacement therapy (HRT) is often offered to protect older women from the undesirable side effects of menopause. Evidence indicative of an associated higher incidence of breast and endometrial cancer and of a higher risk for coronary heart disease and thromboembolism [Rossouw et al., 2002] has questioned the safety of long-term use of estrogen. This led to the search for new substitutes with comparably effective estrogenic activity but without or at least with fewer side effects.

Selective estrogen receptor modulators (SERMs) are well established as synthetic estrogen substitutes [Goldstein et al., 2000]. They operate as estrogen agonists in bone and serum [Meunier et al., 1999] and as antagonists in breast and uterus [Ashby et al., 1997; Muchmore, 2000]. In spite of the widespread use of SERM in post-menopausal women, some mechanisms of SERM activity are still not well defined, such as its influence on the skin and its effect on some of the inflammatory cytokines.

N-3 long-chain fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are often recommended as nutritional additives. Diets rich in these polyunsaturated fatty acids (PUFA) are reported to have a wide variety of beneficial biological functions, such as decreasing serum lipids levels [Saldeen et al., 1998; Mori et al., 2000; Stark et al., 2000], suppressing inflammatory responses [Blok et al., 1997; Grimble, 1998; James et al., 2000] and improving skeletal health [Schlemmer et al., 1999; Watkins et al., 2001a,b]. Dietary n-3 fatty acids are rapidly incorporated into cell membranes [Alexander, 1998; Watkins et al., 2000] and affect membrane fluidity, formation of receptors and ligands binding, as well as activation of intracellular signaling pathways through formation of eicosanoids, gene expression [Jump and Clarke, 1999] and cell differentiation [Alexander, 1998]. Fish oil intake results in partial replacement of arachidonic acid (AA) by EPA/DHA, leading to decreased production of AA-derived mediators (e.g., PGE₂ and LTH₄), an increase in mediators which often have different biological action (e.g., PGE₃ and LTH₅) [Alexander, 1998] and the down-regulation of cyclooxygenase-2 activity [Watkins et al., 1996].

Extensive studies have been carried out on the ability of n-3 fatty acids to influence the

process of cytokines production and the ability of target tissues to respond to these cytokines [Blok et al., 1997; Grimble, 1998; James et al., 2000].

Many activities of n-3 PUFA were shown to parallel the effects that were observed following HRT. This led to the speculation that the addition of n-3 fatty acids to SERM could have more beneficial effects on post-menopausal symptoms than the use of SERM alone.

The aims of this study were to investigate (1) the influence of dietary n-3 PUFA and SERM on serum lipids, skin histology B-lymphopoiesis, and inflammatory cytokines and (2) the synergism between PUFA and SERM in short-term (12–14 weeks) or prolonged (20–24 weeks) estrogen deficiency. We used a murine OVX model to study the results of chronic administration of PUFA separately and in combination with two different SERMs, the raloxifene analogs LY139481 and LY353381.

MATERIALS AND METHODS

Animals

Female balb/c mice aged 6–7 weeks (Animal Laboratories, Tel-Aviv University, Israel) were sham-operated (sham) or ovariectomized (OVX) under general anesthesia using ketamine/rompon solution (0.25 ml/mouse) according to request no. M-98-025 approved by the “Animal Care and Use” Committee of Tel-Aviv University.

Treatment Diets

The mice were fed a basic “Rodent formulab diet” (Purina Test Diets, Richmond, IN) or basic diet supplemented with SERM (Lilly Research Laboratories, Indianapolis, IN) or PUFA (Table I). SERM-treatment diets contained either 0.0013% SERM-I (LY139481) in experiments 1 and 2 or 0.0013% SERM-II (LY353381) in experiment 3. N-3 PUFA-treatment diets consisted of 0.55% menhaden fish oil (EPA/DHA = 2/1) as the source of PUFA. The combined treatment diets contained both SERM (0.0013%) and PUFA (0.55%). Daily consumption was ~3 mg/kg/day of SERM and ~1.25 g/kg/day of PUFA. All diets contained a total of 7.55% fat and 125 ppm Ethoxyquin as an anti-oxidant. The diets were air-dried and vacuum-packed and kept in a dark cool room.

TABLE I. Experimental Groups According to Diets and Surgical Procedures

Group	Surgery	Treatment
Sham	Sham	Basic diet
Sham + SERM	Sham	Basic diet supplemented with SERM-I or SERM-II
Sham + PUFA	Sham	Basic diet supplemented with PUFA
Sham + SERM + PUFA	Sham	Basic diet supplemented with SERM-I or SERM-II
OVX	OVX	Basic diet
OVX + SERM	OVX	Basic diet supplemented with SERM-I or SERM-II
OVX + PUFA	OVX	Basic diet supplemented with PUFA
OVX + SERM + PUFA	OVX	Basic diet supplemented with SERM-I or SERM-II and PUFA

Please see text for definitions.

Experimental Design

Three experiments were performed (A, B, and C) according to two protocols (I and II). There were eight experimental groups in each experiment (Table I), with each group consisting of eight animals.

Experiment A followed protocol I (preventive) in which experimental diets started immediately after OVX. One week after the operation, the mice were divided into groups, allowed free access to the special diets for 12 weeks and then sacrificed (Fig. 1 protocol I). The mice were 20–21 week old at the end of experiment I. Experiments B and C followed protocol II (curative) in which treatment started at 13 weeks post-OVX. The operated mice were kept on a control diet for 13 weeks following surgery and then divided into eight treatment groups. They were allowed free access to the special diets (SERM-I in experiment B and SERM-II in experiment C) for 12 weeks and then sacrificed (Fig. 1 protocol II). The mice were 35–36 weeks old at the end of experiment II.

Body Weight

All the animals were weighed before the operation, before initiating the experimental diets, and at the end of each experiment.

Uterus Weight

The uteri were fixed with 4% formaldehyde and air-dried, after which they were weighed.

Skin Dermis and Adipose Layer Thickness

Skin samples were obtained from the low thoracic/upper lumbar areas. After removing the hair, the samples (1 × 1 cm) were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 72 h and embedded in paraffin blocks. The slides were stained with hematoxylin–eosin. Images of skin sections at a magnification of ×40 were captured by a Pixera TV camera connected to a Macintosh Power PC 63 computer using the software provided by the Pixera Company. At least 3–5 measurements of dermis and adipose layers thickness were performed on each section of skin.



Fig. 1. Experiment A: Followed the preventive protocol (protocol I) in which experimental diets started immediately after ovariectomy (OVX). One week after the operation, the mice were divided into groups, allowed free access to the special diets for 12 weeks and then sacrificed. Experiments B and C followed

protocol II (curative) in which treatment started at 13 weeks post-OVX. The operated mice were kept on a control diet for 13 weeks following surgery and then divided into eight treatment groups. They were allowed free access to the special diets for 14 weeks and then sacrificed.

Serum Biochemistry

Blood samples were obtained at sacrifice and allowed to clot overnight at 4°C. Serum samples from mice in the same group were pooled and kept in aliquots at -20°C. Calcium (Ca), magnesium (Mg), inorganic phosphorous (Pi), glutamic-oxaloacetic transaminase (GOT), and glutamate-pyruvate transaminase (GPT), were analyzed in serum samples by an automatic analyzer (Boehringer-Hitachi 747). Alkaline phosphatase (AP) was measured using Boehringer-Mannheim analytical kits.

Triglycerides concentration was analyzed enzymatically. A high-performance colorimetric assay was utilized for cholesterol quantification. Serum high-density lipoprotein (HDL) was determined by precipitation with phosphotungstate using a Boehringer-Mannheim kit.

Bone Marrow Cells (BMC)

BMC were flushed from the femora and tibiae in a minimal volume (1-3 ml) of Dulbecco's minimum essential medium (DMEM) containing 5% fetal calf serum (FCS), pooled and centrifuged. The BMC were separated from marrow plasma as described earlier [Kassem et al., 1996; Vargas et al., 1996], resuspended in DMEM and kept on ice.

Splenocytes

Spleens collected in DMEM were mechanically smashed. The single-cell suspension was resuspended and counted. Using a flow analysis cytometry system (FACS, Becton Dickinson immunocytometry system, Inc., San Jose, CA), 10⁶ BMC or splenocytes were stained with specific fluorescein isothiocyanate (FITC)-conjugated antibodies against cell surface antigens. Cells were stained for 1 h at 4°C, washed twice with buffer containing 5% FCS and 0.1% azide in PBS, and fixed with 2% paraformaldehyde in PBS containing 0.1% azide. The stained cells were quantitatively analyzed by FACS. The 488 nm beam was used for excitation and the FITC fluorescence emission was measured at 510-530 nm.

We used several antibodies to differentiate between bone-marrow subpopulations: anti-CD 45R (B-220) which is specific staining for B cells (Southern Biotechnology Associates, Inc., Birmingham, AL), CD-11b (mac-1) which recognizes mainly monocytes and macrophages (Sigma F-2648, St. Louis, MO), CD-90 (Thy-1.1)

which is specific for T cells (Sigma F-2887), Ly-6G (Gr-1) anti-granulocytes (Pharmingen 01214A, San Diego, CA), and CD-31-stained endothelial cells (Pharmingen).

Bone Marrow: Cytokine Analysis In Vivo (Marrow Plasma) and Ex Vivo (Conditioned Medium)

For cytokine analysis, BMC were flushed from the femora and tibiae with cold DMEM containing 5% FCS. Marrow plasma and cells were treated separately. The marrow plasma was immediately frozen in aliquots and kept at -80°C for further in vivo cytokine analysis. Five aliquots of 10⁷ cells were used for ex vivo (conditioned medium (CM)) analysis. The cells were seeded in 24-well plates in DMEM either with or without 5 µg/ml of lipopolysaccharides (LPS) (Sigma L-2630) for 20 h (Table II). The CM was collected and pooled, and aliquots were kept at -80°C for cytokine analysis. Non-induced cytokine secretion was analyzed first. LPS-induction was performed in samples with undetectable cytokines levels (Table II).

Cytokine content in CM and in marrow plasma was determined using an immunoassay technique. IL-10 (4-1,000 pg/ml), IL-1β, TNF-α (5-1,500 pg/ml), and IL-6 (3-1,000 pg/ml) were quantified using Quantikine[®] M kits (R&D systems, Inc., Minneapolis, MN).

Statistical Analysis

The statistical analyses were performed using SPSS for Windows. The data were expressed as the mean ± SEM of at least three measurements. The differences between groups were analyzed by one-way analysis of variance (ANOVA). When the ANOVA test showed significant ($P < 0.05$) differences among the groups, statistical differences between every two groups were evaluated by means of the Duncan multiple comparison test.

TABLE II. Constitutive or Induced Bone-Marrow Cytokines Analysis

Cytokine secretion	Constitutive secretion	Induced secretion
	-LPS	+LPS
TNF-α	+	Not done
IL-6	+	Not done
IL-1β	Not detectable	+
IL-10	Not detectable	+

LPS, lipopolysaccharides (5 µg/ml).

RESULTS

Effects of SERM and PUFA on Body and Uterine Weight

In all the experiments, the body weight of the OVX animals significantly increased compared to the sham controls. In experiment A, weight gain of the growing animals during the initial 12 weeks of the experiment was significantly inhibited ($P < 0.05$) upon receiving SERM-I both in the sham and OVX animals (data not shown). The PUFA-containing diet significantly ($P < 0.05$) interfered with weight gain in the sham but not in the OVX animals. In contrast, there were no significant differences in body weight at the end of experiments B and C (33–35 weeks from onset of treatment).

OVX was associated with a decreased uterine weight in all experiments (by 55–80%, $P < 0.05$). Neither SERM nor PUFA, nor the combination of both had any effect on uterine involution in the OVX groups (Fig. 2). In both protocols, SERM-I administration (Fig. 2A,B) interfered with normal uterine growth in the sham mice: 12 weeks of treatment yielded a markedly lower uterine weight (37–40% that of sham controls, $P < 0.05$). The effect with SERM-II was not significant (Fig. 2C).

Effects of SERM and PUFA on Skin Dermis and Adipose Thickness

OVX significantly raised (by 28–40%, $P < 0.05$) the total thickness of skin (Figs. 3 and 4). The 45–64% increase in the adipose tissue's thickness was responsible for most of the elevation in the total skin thickness, while the dermis was not significantly thicker (Fig. 5 panels I, II). SERM reduced the thickness of adipose and dermis tissues dramatically, i.e., by 48–60 and 15–25%, respectively, ($P < 0.05$) and

overcame the effect of OVX (Fig. 4A–C). A 12-week consumption of PUFA caused a 30–40% decline in the adipose thickness in the OVX groups but, in contrast to SERM, PUFA had no effect on the dermis. The decreased adipose and dermis thickness in the OVX mice that received combined PUFA and SERM mimicked the activity of SERM given alone. We saw no positive effect of fish oil or SERM on the appearance of skin hair or number of hair follicles in the OVX animals or differences in the immunohistochemical staining intensity for collagen type I (data not shown).

Serum Biochemical Parameters in Response to the Addition of SERM and PUFA

OVX increased the concentration of serum lipids (cholesterol and triglycerides) as had already been shown earlier in many cases [Walsh et al., 1998; Stark et al., 2000]. Both SERM derivatives, SERM-I and SERM-II, prevented the elevation of serum lipids that had been caused by estrogen withdrawal (Fig. 5). Following treatment with both derivatives of SERM, total cholesterol and triglycerides levels decreased by 18–38% in the sham group and by 0–40% in the OVX group. A 12-week consumption of PUFA was effective in decreasing the triglyceride levels by 14–44% in the OVX mice (Fig. 5A–C), but it had a negligible effect on cholesterol (Fig. 5 panel II) or HDL (data not shown). Although PUFA and SERM given separately did lead to a decrease in the levels of triglycerides, they had no additive effect in the mice that received both compounds in their diet.

There was no difference in the effect of SERM and PUFA on liver function (GOT, GPT) and serum chemistry (Ca, Mg, Pi, and AP) between the treatment and control mice. All values were within normal range (data not shown).

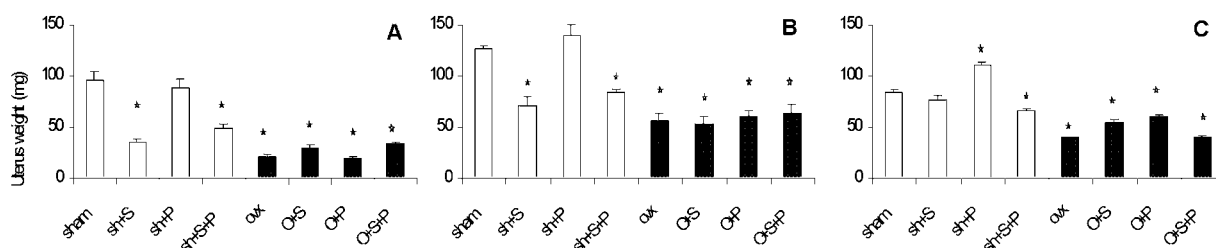
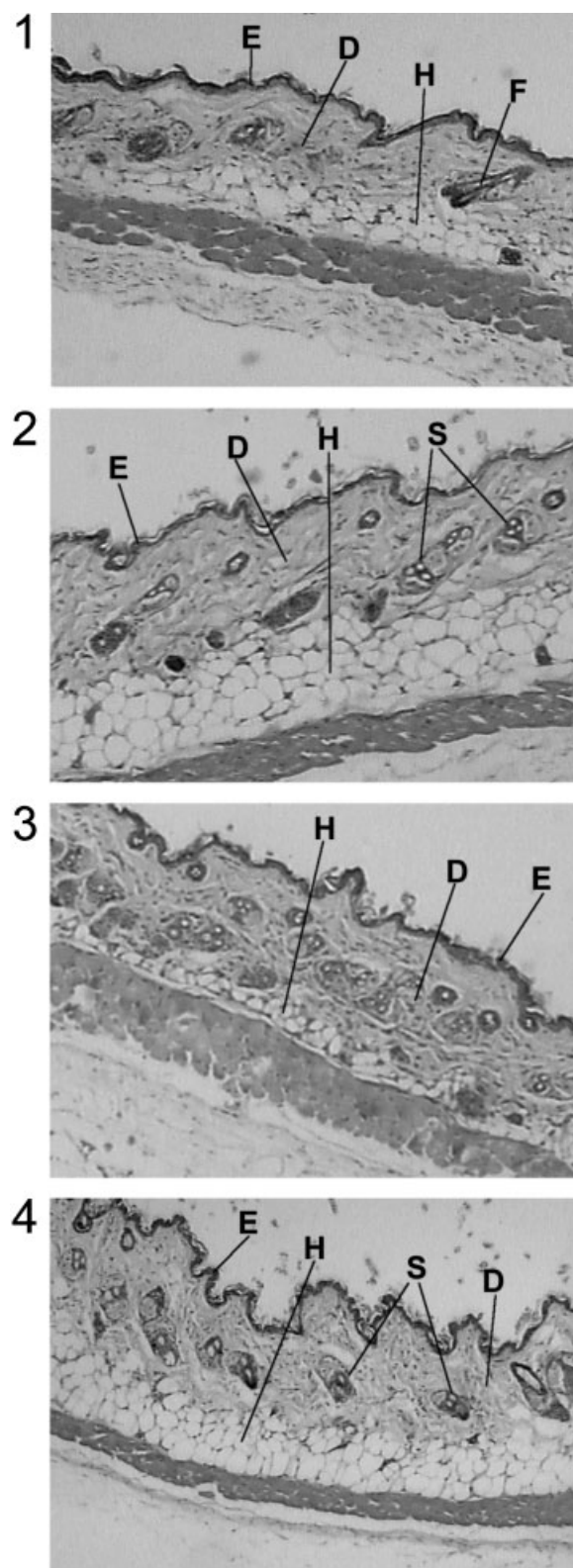


Fig. 2. Effect of polyunsaturated fatty acids (PUFA) (P), selective estrogen receptor modulator (SERM) (S)-I [experiments A, B], and SERM-II [experiment C] in sham-operated (sh) (empty bars) or OVX (solid bars) on uterus wet weight. A: Oral administration of

SERM (0.0013%)/PUFA (0.55%) started 1 week postoperatively and lasted for 12 weeks (protocol I), (B, C) oral administration started 12 weeks post-OVX for 12 weeks (protocol II). Results are mean \pm SE (* $P < 0.05$ compared to sham controls).



FACS Analysis of BMC

We analyzed the expression of specific antigens in cells of the hemopoietic compartments of the bone marrow and spleen, including T and B cells, endothelial cells, monocytes/macrophages, and granulocytes. Estrogen depletion affected bone-marrow cellular composition in growing animals (experiment A). We detected a 20% ($P < 0.05$) decrease in the Gr-1-positive population and a 36–65% ($P < 0.05$) increase in the number of T cells, while there was no effect on the monocytes/macrophages population (data not shown).

We also observed a decrease of 37–55% ($P < 0.05$) in endothelial cells from the spleen but not from the bone marrow (data not shown). Having not detected any PUFA or SERM effects on these cell populations, we did not analyze them in experiments B and C.

An up-regulation of 62% ($P < 0.05$) in the B cells positively stained with CD-45R mAb was detected at 12 weeks post-OVX (Fig. 6A). Prolonged estrogen deficiency (experiments B and C) caused only a 16–28% ($P < 0.05$) elevation in B-lymphopoiesis (Fig. 6B,C). In the PUFA-fed animals, B-lymphopoiesis decreased when treatment was started immediately post-OVX (prevention, Fig. 6A) but not in the animals included in the long-term protocol (curative, Fig. 6B,C). The presence of SERM-II slightly raised B-lymphopoiesis in both the sham and OVX mice (Fig. 6C), while SERM-I had no effect upon it. The addition of SERM-I to n-3 PUFA in the short-term experiment had a synergistic effect. The combined effect of SERM-II and PUFA reflected their individual effect.

Bone-Marrow Cytokine Analysis—In Vivo

IL-1 β . At 24 weeks post-OVX, the IL-1 β levels in the bone marrow plasma were elevated by 23–51% compared to the sham controls (Fig. 7B,C pane I). The increased content of n-3 PUFA in the diet spared the mice from the OVX effect and decreased the levels of IL-1 β to

Fig. 3. Skin sections ($\times 40$) stained with hematoxylin-eosin from sham control [1], OVX [2], OVX mice receiving SERM-I [3], and OVX mice receiving n-3 PUFA [4]. Total thickness of the dermis (epidermis [E] + dermis [D]) and the hypodermis [H] which consist largely of adipose tissue were measured in skin sections taken from mice in experiment A. Sweat glands [S] and hair follicles [F] are located in the dermis and hypodermis.

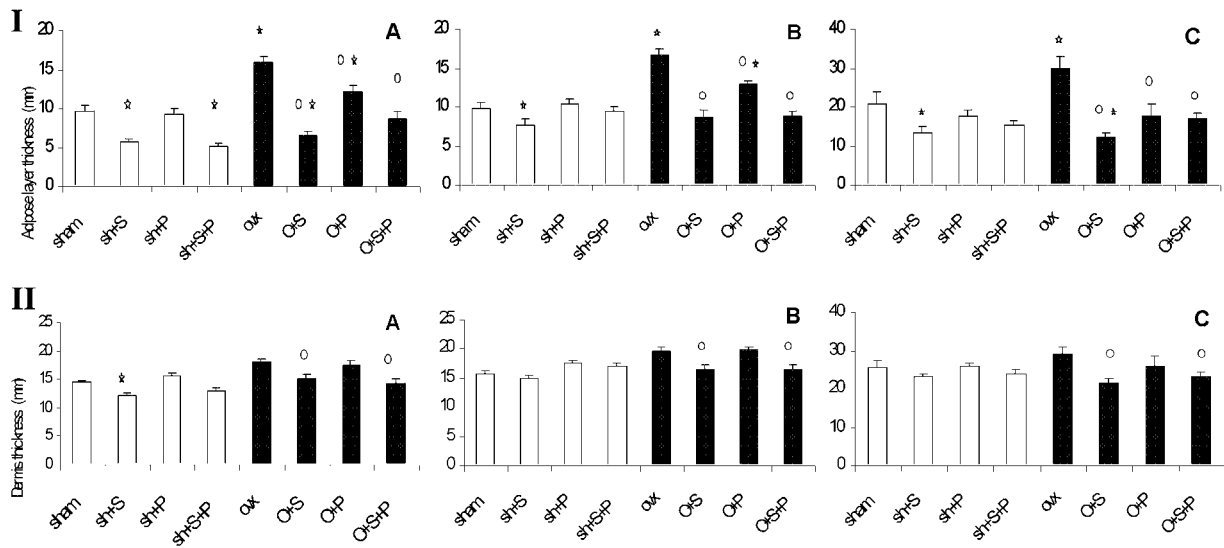


Fig. 4. The effect of PUFA (P), SERM (S)-I [experiments A, B], and SERM-II [experiment C] in sham-operated (sh) (empty bars) or OVX (solid bars) on the thickness of skin adipose tissue (**panel I**) and on skin dermis thickness (**panel II**). **A:** Oral administration of SERM (0.0013%)/PUFA (0.55%) starting 1 week postoperatively

and lasting for 12 weeks (protocol I), (**B, C**) oral administration of the same regimen starting at 12 weeks post-OVX and lasting for 12 weeks (protocol II). Results are mean \pm SE (* P < 0.05 compared to sham-operated control; ° P < 0.05 compared to OVX control).

that of the sham controls. The influence of PUFA was also seen in the sham-operated mice but the change failed to reach statistical significance.

Although SERM supplement alone had no effect, the combined PUFA and SERM treatment decreased IL-1 β levels more than PUFA alone.

Since the IL-6, IL-10, and TNF- α levels in bone marrow plasma were barely detectable,

these measurements were not suitable for statistical assessment.

Cytokine Production in CM From Cultured BMC

IL-1 β . Some of the results from ex vivo (cultured BMC) measurements were unexpectedly opposite from those obtained from in vivo analysis (marrow plasma). Estrogen deficiency

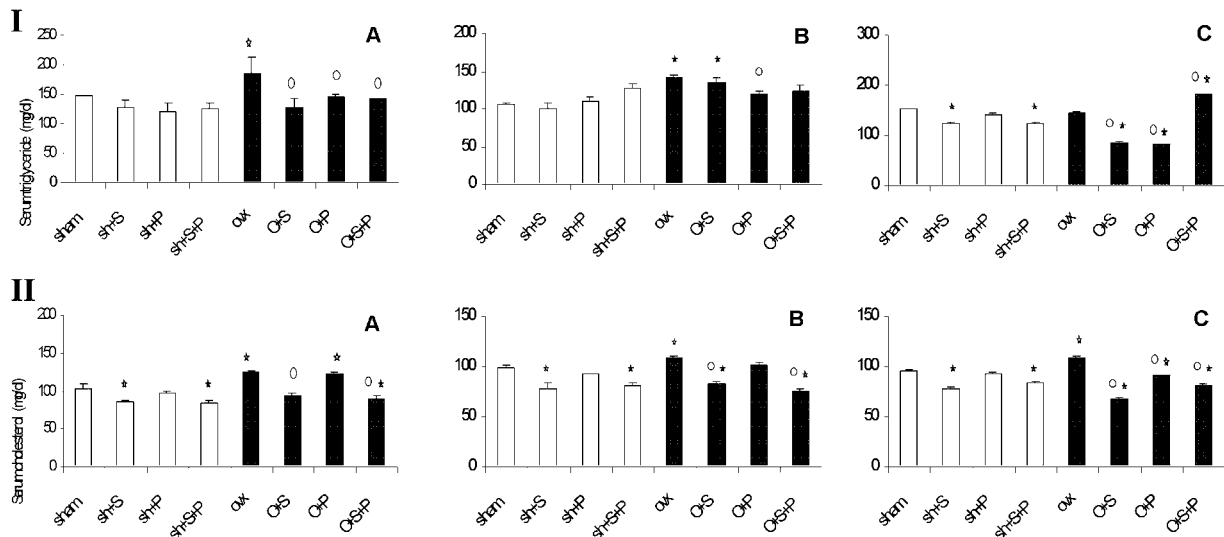


Fig. 5. The effect of PUFA (P), SERM (S)-I [experiments A, B], and SERM-II [experiment C] in sham-operated (sh) mice (empty bars) or OVX mice (solid bars) on serum triglycerides (**panel I**) and serum total cholesterol (**panel II**). **A:** Oral administration of SERM (0.0013%)/PUFA (0.55%) starting at 1 week postoperatively and

lasting for 12 weeks (protocol I), (**B, C**) oral administration starting at 12 weeks post-OVX and lasting for 12 weeks (protocol II). Results are mean \pm SE (* P < 0.05 compared to sham-operated controls; ° P < 0.05 compared to OVX controls).

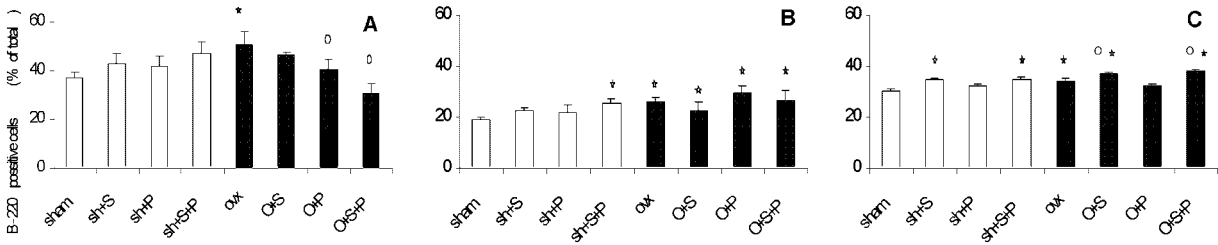


Fig. 6. The effect of PUFA (P), SERM (S)-I [experiments A, B], and SERM-II [experiment C] in sham-operated (sh) mice (empty bars) or OVX mice (solid bars) on B-lymphopoiesis. Pooled bone marrow cells (BMC) were stained with FITC-conjugated CD-45R, analyzed by flow-cytometry and the percentage of positive cells was calculated. **A:** Oral administration of SERM (0.0013%/

PUFA (0.55%) starting at 1 week postoperatively and lasting for 12 weeks (protocol I), **(B, C)** oral administration of SERM (0.0013%/PUFA (0.55%) starting at 12 weeks post-OVX and lasting for 12 weeks (protocol II). Results are mean \pm SE (* P < 0.05 compared to sham-operated controls; ° P < 0.05 compared to OVX controls).

had no effect on ex vivo IL-1 β secretion (Fig. 7 panel II) while causing a small increase in IL-1 β levels in marrow plasma (Fig. 7 panel I). PUFA elevated LPS-induced IL-1 β secretion into CM by 14–61% (P < 0.05) in contrast to the decreased amount of IL-1 β measured in vivo in animals receiving n-3 PUFA in their diet. Although SERM given alone has increased IL-1 β secretion, the combined treatment of SERM and PUFA has no effect when compared to OVX animals.

IL-6. Estrogen withdrawal did not alter the non-induced secretion of IL-6 into the medium of cultured BMC (Fig. 8B,C). The treatment of

OVX animals with PUFA significantly decreased the cytokine secretion by 26–64% (P < 0.05). The effect of PUFA was also seen in the sham-operated mice but the results did not reach a level of statistical significance. Neither SERM-I nor SERM-II changed the potential of the cells to secrete IL-6 in the sham or OVX-operated animals. The effect of PUFA disappeared in mice that received both, SERM and PUFA.

TNF- α . N-3 PUFA significantly lowered constitutive (i.e., non-induced) TNF- α secretion in cultured BMC from both the sham and OVX mice (Fig. 9B,C). The effect of n-3 PUFA was noticed at each measured time point (data not

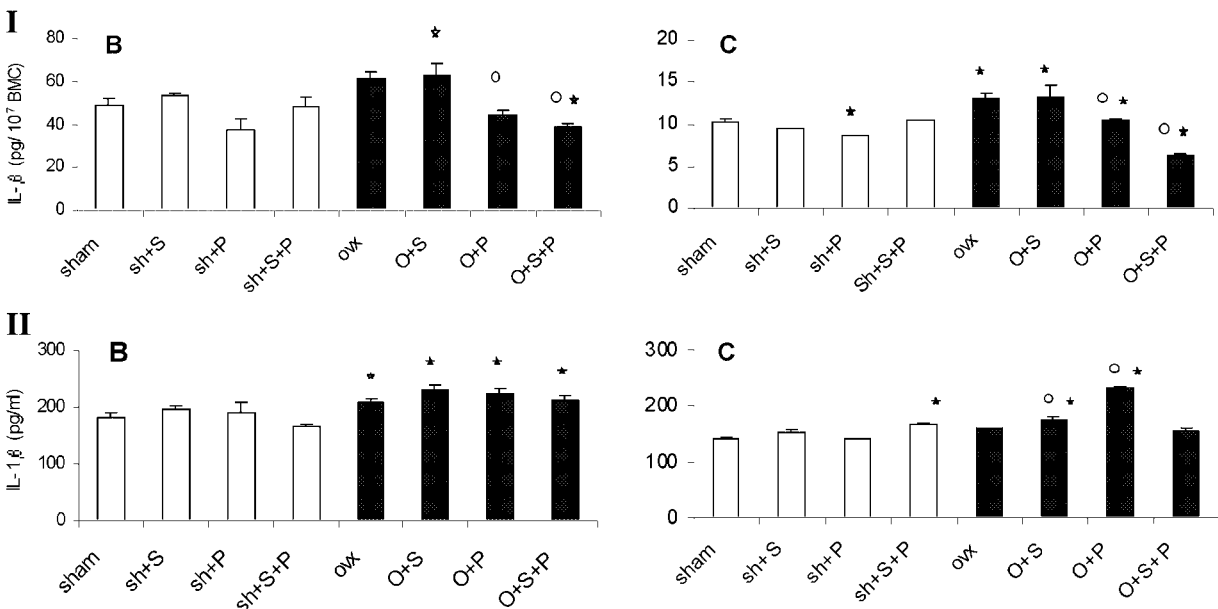


Fig. 7. The effect of PUFA (P), SERM (S)-I [experiment B], and SERM-II [experiment C] in sham-operated (sh) mice (empty bars) or OVX mice (solid bars) on IL-1 β secretion by cultured BMC (2.5×10^7 /ml, 24 h) induced by 5 μ g/ml lipopolysaccharides (LPS) (**panel I**) and IL-1 β amount in bone-marrow plasma

(**panel II**). Oral administration of SERM (0.0013%/PUFA (0.55%) started at 12 weeks post-OVX and lasted for 12 weeks (protocol II). Results are mean \pm SE (* P < 0.05 compared to sham-operated controls; ° P < 0.05 compared to OVX controls).

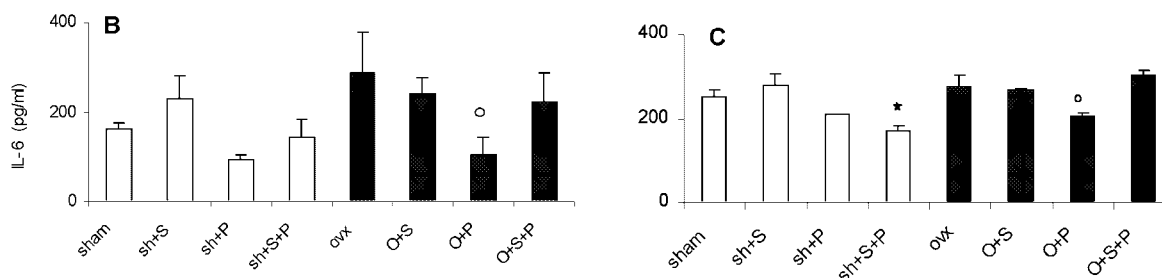


Fig. 8. The effect of PUFA (P), SERM (S)-I [experiment B], and SERM-II [experiment C] in sham-operated (sh) mice (empty bars) or OVX mice (solid bars) on IL-6 secretion by cultured BMC (2.5×10^7 /ml, 24 h). Oral administration of SERM (0.0013%)/

PUFA (0.55%) started at 12 weeks post-OVX and lasted for 12 weeks (protocol II). Results are mean \pm SE (* $P < 0.05$ compared to sham-operated controls; ^o $P < 0.05$ compared to OVC controls).

shown). Chronic administration of SERM-I or SERM-II also decreased TNF- α secretion, but the combined SERM and PUFA treatment canceled the lowering effect of each one when given separately.

IL-10. OVX had no effect on IL-10 amounts secreted from BMC. Treatment with any SERM derivatives failed to change the amount of the cytokine secreted by BMC in either the sham or OVX animals. Induction of IL-10 secretion following chronic administration of n-3 PUFA was detected in both the sham and OVX mice (Fig. 10B,C). There was no stimulatory effect of PUFA in animals fed with the combined PUFA and SERM regimen (SERM-I and SERM-II).

DISCUSSION

Estrogen is involved in the modulation of a variety of functions of many organs, in addition to its activity on the female reproductive system. Post-menopausal women may experience rapid bone loss, elevated serum lipids

levels, and increase in proinflammatory cytokines due to decreased estrogen levels.

The mechanism of estrogen activity is not clearly defined, but it has been found that estrogen up-regulates peroxisome proliferator-activated receptor-gamma (PPAR-gamma) [Ma et al., 1998a,b] and modulates gene transcription by affecting the NFkB pathway [Cerillo et al., 1998; Sanchez et al., 2002]. Transcription factors such as retinoic X receptor (RXR) and PPAR-gamma are capable of activating estrogen-responsive genes in a mechanism that may involve direct binding to the estrogen response elements [Nunez et al., 1997; Sanchez et al., 2002]. Estrogen induces the metabolism of arachidonic acid and thus the production of a PPAR ligand (PGJ₂) in PPAR-gamma-expressing tissues [Ma et al., 1998a,b]. Estrogen has been reported to modulate adipogenesis and was found to up-regulate PPAR-gamma-2 in differentiated pre-adipocytes [Dieudonne et al., 2000], but the role of sex hormones in adipogenesis is still poorly understood.

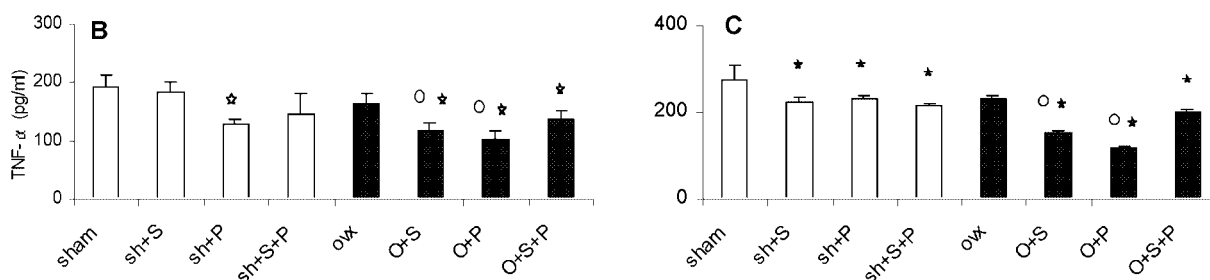


Fig. 9. The effect of PUFA (P), SERM (S)-I [experiment B], and SERM-II [experiment C] in sham-operated (sh) mice (empty bars) or OVX mice (solid bars) on TNF- α secretion by cultured BMC (2.5×10^7 /ml, 24 h). Oral administration of SERM (0.0013%)/PUFA (0.55%) started at 12 weeks post-OVX and lasted for 12 weeks (protocol II). Results are mean \pm SE (* $P < 0.05$ compared to sham-operated controls; ^o $P < 0.05$ compared to OVX controls).

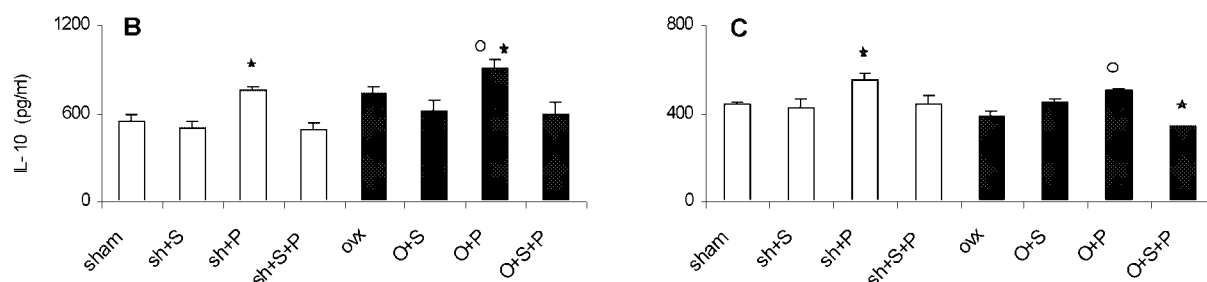


Fig. 10. The effect of PUFA (P), SERM (S)-I [experiment B], and SERM-II [experiment C] in sham-operated (sh) mice (empty bars) or OVX mice (solid bars) on IL-10 secretion by cultured BMC (2.5×10^7 /ml, 24 h) induced by 5 μ g/ml LPS. Oral administration

of SERM (0.0013%)/PUFA (0.55%) started at 12 weeks post-OVX and lasted for 12 weeks (protocol II). Results are mean \pm SE (* $P < 0.05$ compared to sham-operated controls; ° $P < 0.05$ compared to OVX controls).

SERMs have been studied in depth and are in wide use among post-menopausal women as estrogen substitutes [Clemett and Spencer, 2000; Muchmore, 2000; Prestwood et al., 2000]. They act as agonists in some tissues (bone and serum) and as antagonists in others (uterus, breast).

The beneficial effect of n-3 PUFA over n-6 PUFA was shown by many authors [Camandola et al., 1996; Alexander, 1998; Grimble, 1998; Jump and Clarke, 1999; Roche and Gibney, 2000]. Inflammatory cytokine production is suppressed by n-3 PUFA, while n-6 PUFA exert the opposite effect [Alexander, 1998; Grimble, 1998]. In contrast to short n-6 fatty acid, n-3 PUFA repress genes encoding lipogenic, glycolytic, and cholesterolgenic enzymes and increase expression of genes of enzymes participating in β -oxidation [Jump and Clarke, 1999]. N-3 PUFA exert their different modulatory action on gene expression by changing the profile of eicosanoids, decreasing the oxygenation efficiency of COX-1 [Jump, 2002] and the activation/suppression of transcription factors such as PPAR- α and PPAR- γ , RXR, sterol response element binding protein (SREBP) and AP-1 [Alexander, 1998; Grimble, 1998; Jiang et al., 1998; Jump and Clarke, 1999; Berger and Moller, 2002].

PPAR- α is a key factor in lipids modulation, while PPAR- γ is mainly involved in the immunological responses through prostaglandins and the NF κ B pathway. The finding that PUFA are PPAR ligands [Forman et al., 1997; Thoennes et al., 2000] can explain the beneficial effects of dietary PUFA.

The similarity between PUFA and estrogen in the modulation of gene expression via PPARs and NF κ B (which are widely expressed in many

tissues) motivated the investigation of their in vivo effects on target organs.

In this report, we studied the potential of the dietary addition of n-3 PUFA and SERMs to OVX mice as alternative treatment to HRT. We analyzed these in vivo effects in estrogen target tissues (bone marrow, skin, serum lipids, and uterus) with respect to long-term effects of estrogen deficiency.

Effect of n-3 PUFA and SERM on Serum Lipids

OVX is often accompanied by higher serum cholesterol and triglycerides levels [Walsh et al., 1998; Stark et al., 2000]. Our results showed that both SERM derivatives decreased cholesterol and triglyceride levels in serum of OVX mice as well as that of sham-operated mice. These findings are in contrast to other published results on SERM which demonstrated an inhibitory effect on cholesterol but not on triglycerides [Kauffman et al., 1997; Sato et al., 1998; Walsh et al., 1998; Goldstein et al., 2000; Blum and Cannon, 2001]. The higher dose of SERM used in our model (i.e., ~ 3 mg/kg/day) compared to the dose of 1 mg/kg/day used in most publications, and the fact that our mice consumed SERM in their diet for 8–12 weeks, a much longer period of time than in other experiments, could explain these differences. Interestingly, the inhibitory effect of SERM on serum cholesterol was also detected in the sham-operated mice. This might be explained by either enhanced activity of the SERM–ER or increased availability of the ligand-bound estrogen receptor [Klinge, 2001; Sanchez et al., 2002]. HDL is a key factor in cholesterol synthesis and secretion. ApoA-I and ApoA-II (the major apolipoproteins in HDL) are down-regulated by PPAR- α ligands-like fibrates which

are hypolipidemic drugs [Peters et al., 1997; Jump and Clarke, 1999]. By their activity as estrogen receptor agonists, SERM could exert their inhibitory effect on serum lipids by modulating PPAR- α activity.

Menhaden fish oil (~1.25 mg/kg/day) decreased serum triglycerides but had no effect on cholesterol levels. These results are consistent with those of other studies that showed lower triglycerides levels following n-3 PUFA consumption [Saldeen et al., 1998; Mori et al., 2000; Roche and Gibney, 2000; Stark et al., 2000]. N-3 PUFA was shown to modulate both the synthesis of triglycerides and their clearance from the serum [Jump and Clarke, 1999; Jump, 2002]. N-3 PUFA reduced hepatic VLDL synthesis and secretion by inhibition of *ApoCIII* gene transcription through the inhibition of PPAR- α and hepatic nuclear factor-4 (HNF4) binding to the promoter of the *ApoCIII* gene [Jump and Clarke, 1999; Mori et al., 2000]. Lipoprotein lipase (LPL) hydrolyzes triglycerides from circulating lipoproteins, thus lowering the amount of triglycerides in the serum. It was also shown that the PPAR- α responsive element in the LPL promoter is under the regulation of PUFA [Schoonjans et al., 1996]. Our results demonstrated that SERM and/or n-3 PUFA were effective in decreasing serum lipids as preventive agents or as an effective treatment of established chronic estrogen deficiency.

Effect of n-3 PUFA and SERM on Skin

The effect of SERM or n-3 PUFA on skin has been poorly studied. PUFA have been shown to influence wound healing and irradiation protection of skin through NF κ B [Kaufman and Fuchs, 2000; Boelsma et al., 2001]. Receptors for estrogen have been identified in the epidermis, dermis, and blood vessels of skin [Brincat, 2000], but the exact mechanism by which estrogen influences the skin cellular composition and function is not known. In our study, SERM and PUFA inhibited the increase in adipose tissue thickness caused by OVX. This effect was probably secondary to their effect on decreasing serum triglycerides concentration, and it was associated with lowering of body weight. The effects of SERM on the subcutaneous adipose layer may also indicate an involvement of estrogen receptors in local modulation of lipid metabolism and/or accumulation, in the skin. The effect of n-3 PUFA could be also attributed to PUFA's activity

through PPAR- γ : as had been shown by [Rubin et al., 2000], activation of PPAR- γ by troglitazone inhibited the cytokines-induced expression of aromatase in human breast adipocytes, thereby causing a decrease in adipogenesis.

Effect of n-3 PUFA and SERM on Cytokine Secretion

Hypoestrogenism is accompanied by elevated levels of inflammatory cytokines [Editorial, 1998; Rodan and Martin, 2000]. ER modulates genes transcription by affecting the NF κ B pathway [Cerillo et al., 1998; Clemett and Spencer, 2000; Sanchez et al., 2002] which is a central transcription factor in cytokine synthesis and activity. Genes such as *COX2*, *TNF- α* , and *IL-6* have binding sites for NF κ B and are activated following its binding.

TNF- α and IL-1 β studies in healthy volunteers and in patients with rheumatoid arthritis have shown as high as a 90% inhibition of cytokine production after 6 weeks of dietary supplementation with fish oil [Blok et al., 1997; James et al., 2000]. Sadeghi et al. [1999] found that plasma TNF- α , IL-1 β , and IL-6 levels were lower in mice which had been fed for 5 weeks with a fish oil-rich diet than those in mice fed safflower oil. Similar results were found in ex vivo experiments in human and rat peripheral blood cells [Somers and Erickson, 1994; James et al., 2000].

In our experiments, OVX had not changed BMC IL-6, TNF- α , IL-1 β , or IL-10 secretion into CM (i.e., the higher levels of cytokines observed in some cases were not found to be statistically significant). These findings contradict published results which demonstrated elevations in the levels of inflammatory cytokines following estrogen withdrawal [Editorial, 1998; Rodan and Martin, 2000]. The fact that SERM also had no affect on IL-6, IL-1 β , or IL-10 secretion gives more credence to the idea that these cytokines are not under the immediate regulation of estrogen. In contrast to the cytokines mentioned above, TNF- α was inhibited by both SERM derivatives. The findings that SERM decreased TNF- α while estrogen withdrawal did not might represent an antagonistic effect of SERM.

PUFA had a lowering effect on IL-1 β levels in bone marrow plasma (in vivo) but it increased the IL-1 β secretion in cultured BMC (ex vivo). These surprisingly opposite results suggest that PUFA does not directly affect IL-1 β via

modulating gene transcription, but rather exerts its inhibitory effect through other post-transcriptional activity or via other cell types existing in the bone marrow. N-3 PUFA decreased secretion of non-induced IL-6 and TNF- α from cultured BMC similarly to other published results [Somers and Erickson, 1994; Blok et al., 1997; Alexander, 1998; Grimble, 1998; Sadeghi et al., 1999].

IL-10 is an anti-inflammatory cytokine. It was shown to suppress TNF- α , IL-6, and other inflammatory cytokines as well as COX-2 in marrow cell subpopulations, such as T cells, monocytes/macrophages and B-cells, by inhibiting the activation of NF κ B [Moore et al., 2001]. Our results revealed a significant elevation in IL-10 secretion following n-3 PUFA consumption.

It was proposed that increased apoptosis of monocytes/macrophages following activation of PPAR-gamma and NF κ B is involved in the decrease of inflammatory cytokines [Camandola et al., 1996; Ricote et al., 1998; Mori et al., 2000; Berger and Moller, 2002]. We saw no change in the monocyte/macrophage population in our FACS analysis of BMC: our results demonstrated that the increased IL-10 levels are probably responsible for the decreased TNF- α and IL-6 in animals receiving n-3 PUFA.

Effect of n-3 PUFA and SERMs on B-Lymphopoiesis

Estrogen is a negative regulator of B-lymphopoiesis [Medina et al., 2000; Onoe et al., 2000]. Two mechanisms for B-lymphopoiesis regulation by estrogen have been suggested: decreased mitotic activity of early B cells in hormonal-treated mice, and increased half-life of mature B cells in estrogen-deficient aged mice. Padilla et al. [2000, 2002] demonstrated increased apoptotic B cells death following activation of PPAR-gamma with PGJ₂. Moreover, early pro-B subpopulations were shown to be dramatically reduced upon estrogen treatment, while differentiated B cells were not affected [Kline et al., 1999; Medina et al., 2000].

In our experiments, unlike the published results of other investigators [Onoe et al., 2000], SERM-I did not decrease the B-lymphopoiesis elevated by OVX. On the other hand, SERM-II exhibited an estrogen-antagonistic effect in the sham-operated and OVX mice, and elevated B-lymphopoiesis. The differences between our results and other published data might stem

from the differences in duration of treatment (12 weeks in our study compared to 2 weeks in other works), the SERM dose (3 mg/kg/day compared to 0.1 mg/kg/day) or the specific SERM derivative.

Fish oil consumption decreased the amount of B-cells in the prevention protocol but had no curative effect if it was started 12 weeks after estrogen withdrawal. Our data revealed that interference with B cell maturation but no effect on the existing mature B cell compartment occurred following n-3 PUFA consumption in OVX mice. Our results suggest that PUFA has an effect similar to estrogen in preventing an increase in B-lymphopoiesis, at least in the preventive protocol. This activity could be mediated via the elevation of IL-10, insofar as it was shown that IL-10 is capable of suppressing B-lymphopoiesis [Onoe et al., 2000].

We have shown that oral consumption of PUFA not only affects lipid homeostasis and inflammation but has a multisystem effect as well. N-3 PUFA can spare some of the deleterious estrogen deficiency results. N-3 PUFA decreased triglycerides concentration in the serum, it uniquely increased IL-10 and it decreased inflammatory cytokines and CD-45R expression. SERM had no effect on IL-10, IL-6, or IL-1b, nor did they affect B-lymphopoiesis, but they did decrease serum triglycerides and cholesterol. We observed no significant beneficial effects of the combined administration of SERM and PUFA. The accumulating evidence that PPARs are expressed in most cell types, that NF κ B has a central role in gene transcription, and that n-3 PUFA has the ability to alter these activities may serve to establish the mechanism responsible for the benefits of n-3 PUFA and thus warrant further investigation.

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