

**Differential effects of arachidonic acid and
docosahexaenoic acid on cell biology and
osteoprotegerin synthesis in
osteoblast-like cells**

by

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SUMMARY

The purpose of the study was to elucidate the mechanisms by which polyunsaturated fatty acids (PUFAs) prevent bone loss. MG-63 human osteoblasts and MC3T3-E1 murine osteoblasts were exposed to the n-6 PUFA arachidonic acid (AA) and the n-3 PUFA docosahexaenoic acid (DHA) as well as oestrogen (E2) and parathyroid hormone (PTH) and the effects thereof tested on a variety of biological parameters characteristic of osteoblasts. These parameters included prostaglandin E₂ (PGE₂) synthesis, proliferation, differentiation to mature mineralising osteoblasts as well as osteoprotegerin (OPG) and receptor activator of nuclear factor κ B ligand (RANKL) secretion.

Results showed that AA stimulates PGE₂ production significantly in both cell lines. Stimulated PGE₂ production by MC3T3-E1 cells however, was significantly higher, which might be attributed to auto-amplification by PGE₂ itself in this cell line. Pre-incubation of the MG-63 cells with cyclo-oxygenase (COX)-blockers inhibited PGE₂ production significantly, suggesting that both COX enzymes were involved in PGE₂ synthesis.

The number of functional osteoblasts is important for bone formation therefore *in vitro* osteoblastic cell proliferation was investigated. In contrast to the hormones E2 and PTH, both AA and DHA inhibited proliferation significantly. The AA-mediated anti-proliferative effect is possibly independent of PGE₂ production, as PGE₂ *per se* had little effect on proliferation. DHA inhibited proliferation of MG-63 cells more severely, which might be attributed to the osteosarcoma nature of the MG-63 cells. The anti-proliferative effect of these PUFAs might be attributed to modulation of cell cycle progression or anti-mitotic effects of PUFA peroxidation products. Morphological studies showed apoptotic cells after DHA exposure in MG-63 cells.

There is a reciprocal relationship between reduced proliferation and the subsequent induction of cell differentiation *in vitro*. High basal levels of alkaline phosphatase (ALP) activity, a marker of the mature mineralising osteoblastic phenotype, were detected in MC3T3-E1 cells. Long-term exposure to AA inhibited ALP activity in these cells. This process might be PGE₂-mediated. Exposure to PUFAs, however, did not compromise the ability of the MC3T3-E1 cells to differentiate to mature mineralising osteoblasts.

In contrast with MC3T3-E1 cells, MG-63 cells demonstrated low basal ALP activity and were unable to differentiate to mature mineralising osteoblasts. In the absence of osteogenic-inducing supplements, PUFAs induced adipocyte-like features that might be due to the expression of high levels of PPAR γ in this cell line. Lipid-filled vacuoles were absent in the MC3T3-E1 cells suggesting that the MC3T3-E1 cell line may not express PPAR γ mRNA.

The study furthermore demonstrated that PUFAs are able to modulate OPG and RANKL secretion in osteoblasts. AA inhibited OPG secretion dose-dependently in both cell lines, this could be PGE $_2$ -mediated. AA dose-dependently stimulated soluble RANKL (sRANKL) secretion in MC3T3-E1 cells thereby affecting the OPG/RANKL ratio in a negative way, supporting various reports that AA and PGE $_2$ do cause bone resorption. No sRANKL could be detected after exposing the MC3T3-E1 cells to DHA suggesting that DHA could be protective to bone.

In conclusion, contrary to *in vivo* evidence, this *in vitro* study could not indisputably demonstrate protective effects of PUFAs on the osteoblastic cell lines tested.

KEY WORDS:

Osteoblasts, polyunsaturated fatty acids, arachidonic acid, docosahexaenoic acid, prostaglandin E $_2$, proliferation, differentiation, alkaline phosphatase activity, mineralisation, transdifferentiation, osteoprotegerin (OPG), receptor activator of nuclear factor κ B ligand (RANKL).

OPSOMMING

Die doel van die studie was om die meganismes waardeur poli-onversadigde vetsure (POVS) beenverlies voorkom te verklaar. MG-63 menslike osteoblaste en MC3T3-E1 muis-osteoblaste is blootgestel aan die n-6 POVS aragidoonsuur (AS) en die n-3 POVS dokosaheksaenoësuur (DHS) sowel as estrogeen (E2) en paratiroïedhormoon (PTH) en die effekte daarvan op 'n verskeidenheid biologiese parameters kenmerkend aan osteoblaste getoets. Hierdie parameters sluit in prostaglandien E_2 (PGE_2) sintese, proliferasie, differensiasie na volwasse mineraliserende osteoblaste sowel as osteoprotegerien (OPG) en reseptor aktiveerder van nukleêre faktor κB ligand (RANKL) sekresie.

AS het PGE_2 -produksie in beide sellyne betekenisvol gestimuleer. Gestimuleerde PGE_2 -produksie was aansienlik hoër by die MC3T3-E1-selle wat moontlik toegeskryf kan word aan outoversterking deur PGE_2 in hierdie sellyn. Voorafblootstelling van die MG-63-selle aan sikloöksegenase (SO)-blokkers het PGE_2 -produksie betekenisvol geïnhibeer, wat op die betrokkenheid van beide SO-ensieme by PGE_2 -sintese kan dui.

Aangesien die aantal funksionele osteoblaste belangrik vir beenvorming is, is die *in vitro* proliferasie van osteoblaste bestudeer. In kontras met die hormone E2 en PTH, het beide AS en DHS proliferasie betekenisvol geïnhibeer. Die inhiberende effek van AS op selproliferasie is waarskynlik onafhanklik van PGE_2 -produksie, aangesien PGE_2 op sigself min effek op selproliferasie gehad het. DHS het proliferasie van MG-63-selle meer geïnhibeer as dié van die MC3T3-E1-selle, wat moontlik aan die tumorigeniese aard van die MG-63-selle toegeskryf kan word. Die anti-proliferatiewe effekte van POVS kan moontlik aan modulering van selsiklusprogressie, of andersins aan antimitotiese effekte van POVS-peroksidasiëprodukte toegeskryf word. Morfolgiese studies het die teenwoordigheid van apoptotiese selle na DHS-blootstelling by MG-63-selle aangetoon.

Daar bestaan 'n omgekeerde verwantskap tussen 'n afname in proliferasie en die daaropvolgende induksie van seldifferensiasie *in vitro*. Hoë basaalvlakke van alkaliese fosfatase (ALF)-aktiwiteit, 'n merker vir die volwasse mineraliserende osteoblastiese fenotipe, is by die MC3T3-E1-selle waargeneem. Langdurige blootstelling aan AS het ALF-aktiwiteit in hierdie selle geïnhibeer, wat moontlik PGE_2 -gemedieerd kan wees. Die vermoë van die MC3T3-E1-selle om na volwasse

mineraliserende osteoblaste te differensieer, is egter nie deur blootstelling aan POVS benadeel nie.

In teenstelling met die MC3T3-E1-selle het die MG-63-selle lae basaalvlakke vir ALF-aktiwiteit getoon en hulle was nie in staat om na na volwasse mineraliserende osteoblaste te differensieer nie. In die afwesigheid van osteogenese-induserende supplemente het POVS adiposiet-agtige eienskappe geïnduseer, wat moontlik aan die uitdrukking van hoë PPAR γ -vlakke in hierdie selle toegeskryf kan word. Die afwesigheid van lipiedvakuole by die MC3T3-E1-selle dui daarop dat hierdie sellyn moontlik nie PPAR γ bRNS uitdruk nie.

Die studie het verder getoon dat POVS daartoe in staat is om OPG en RANKL-sekresie in osteoblaste te moduleer. AS het OPG-sekresie in beide sellyne op 'n dosisafhanklik wyse geïnhibeer wat moontlik PGE₂-gemedieer kan wees. AS het verder op 'n dosisafhanklike wyse die sekresie van oplosbare RANKL (oRANKL) in MC3T3-E1-selle gestimuleer en dus die OPG/RANKL verhouding negatief beïnvloed. Hierdie bevinding ondersteun verslae dat AS en PGE₂ beenresorpsie kan veroorsaak. Geen oRANKL is na DHS-blootstelling aan MC3T3-E1-selle waargeneem nie wat daarop kan dui dat DHS moontlik beskerming aan been kan bied.

Opsommend, in teenstelling met *in vivo* studies, kon hierdie *in vitro* studie nie bo alle twyfel beskermende effekte van POVS op die osteoblastiese sellyne soos getoets, aantoon nie.

SLEUTELWOORDE:

Osteoblaste, poli-onversadigde vetsure, aragidoonsuur, dokosaheksaenoësuur, prostaglandien E₂, proliferasie, differensiasie, alkaliese fosfatase-aktiwiteit, mineralisasie, transdifferensiasie, osteoprotegerien (OPG), reseptor aktiveerder van nukleêre faktor κ B ligand (RANKL).

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CONTENTS

Summary.....	i
Opsomming.....	iii
Acknowledgements.....	v
List of Figures.....	xi
List of Tables.....	xiv
List of Abbreviations.....	xv

CHAPTER 1

General Introduction.....	1
---------------------------	---

CHAPTER 2

Literature Review.....	6
I Bone Homeostasis.....	6
2.1 Introduction.....	6
2.2 Composition of bone.....	6
2.3 Bone cells.....	8
2.3.1 Osteoblasts.....	8
2.3.1.1 Origin of osteoblasts.....	9
2.3.1.2 Transcriptional control of osteoblast differentiation.....	10
2.3.2 Osteocytes.....	12
2.3.3 Osteoclasts.....	13
2.3.3.1 Origin of osteoclasts.....	13
2.3.3.2 Regulation of osteoclast differentiation and activation by the osteoprotegerin-RANK-RANKL system.....	15
2.3.4 Cell proliferation and cell death.....	17
2.4 Bone remodeling.....	19
2.4.1 Resorption.....	21
2.4.2 Reversal.....	22
2.4.3 Formation and mineralisation.....	23
2.5 Regulation of bone remodeling.....	23
2.5.1 Circulating hormones.....	23
2.5.1.1 Oestrogen (17 β -estradiol).....	24
2.5.1.2 Parathyroid hormone (PTH).....	26
2.5.1.3 1,25-Dihydroxy vitamin D ₃ (calcitriol).....	28
2.5.2 Prostaglandin E ₂	28
2.5.3 Growth factors and cytokines.....	29
2.6 Summary.....	31

II	Polyunsaturated Fatty Acids	34
2.7	Types of polyunsaturated fatty acids (PUFAs).....	34
2.8	Metabolic pathways of essential fatty acids.....	34
2.9	Cellular functions of polyunsaturated fatty acids.....	35
2.9.1	Composition of membranes.....	36
2.9.2	Eicosanoid synthesis.....	36
2.9.3	Second messengers.....	38
2.9.4	Modulation of gene transcription.....	40
2.10	Effects of polyunsaturated fatty acids on bone.....	42
2.10.1	Essential fatty acid deficiencies and bone.....	42
2.10.2	Nutritional <i>in vivo</i> human and animal studies.....	43
2.10.2.1	Effects of dietary polyunsaturated fatty acids on calcium balance and bone status.....	44
2.10.2.2	Effects of dietary polyunsaturated fatty acids on prostaglandin secretion and bone status.....	45
2.10.2.3	Effects of dietary polyunsaturated fatty acids on insulin-like growth factor (IGF-I) and insulin-like growth factor binding proteins (IGFBPs).....	47
2.10.3	Effects of polyunsaturated fatty acids on bone cells.....	48
2.10.3.1	Effects of polyunsaturated fatty acids on early osteoblastic differentiation.....	48
2.10.3.2	Effects of polyunsaturated fatty acids on osteoclastogenesis.....	49
2.10.3.3	Effects of polyunsaturated fatty acids on cytokine expression.....	50
2.10.3.4	Effects of polyunsaturated fatty acids on alkaline phosphatase (ALP) activity.....	50
2.11	Prostaglandins in bone.....	51
2.11.1	Prostaglandin receptors.....	52
2.11.2	Regulation of prostaglandin production in bone.....	52
2.11.2.1	Stimulation of prostaglandin production in bone.....	52
2.11.2.2	Inhibition of prostaglandin production in bone.....	54
2.11.3	<i>In vitro</i> effects of prostaglandin E ₂ on bone.....	55
2.11.3.1	<i>In vitro</i> effects of prostaglandin E ₂ on bone formation.....	55
2.11.3.2	Prostaglandin E ₂ effects on bone resorption.....	58
2.12	Summary.....	60
CHAPTER 3		
	General Cell Culture Procedures	62
3.1	Cell cultures.....	62
3.1.1	Cell lines.....	62
3.1.2	Maintenance of cell cultures.....	62
3.1.3	Choice and preparation of cell culture media.....	64
3.1.3.1	Preparation of cell culture media for oestrogen exposure....	64
3.1.3.2	Media for growth (proliferation) studies.....	64
3.1.3.3	Osteogenic supplemented media.....	65
3.1.4	Trypan blue exclusion test for cell viability.....	65

3.2	Preparation of stock solutions.....	66
3.3	Standardisation of a method for quantification of cell number.....	67
3.4	Prostaglandin E ₂ -related experiments.....	68
3.4.1	Radioimmunoassay (RIA) of prostaglandin E ₂ in cell culture media.....	68
3.4.2	Indirect immunofluorescence for detection of COX-1 and COX-2 in MG-63 cells.....	70
3.5	Proliferation studies.....	70
3.6	Haematoxylin and eosin (H&E) cell staining.....	71
3.7	Hoechst 33342 (HOE) and propidium iodide (PI) staining for detection of apoptosis.....	72
3.8	Quantification of alkaline phosphatase (ALP) activity.....	73
3.9	Detection of mineralising properties.....	74
3.10	Assay of adipocytogenesis.....	74
3.10.1	Quantification of adipocytogenesis by Oil Red O staining.....	74
3.10.2	Microscopic visualisation of lipid accumulation.....	75
3.11	Measurements of osteoprotegerin and RANKL secretion.....	76
3.11.1	Enzyme linked immunosorbent assay (ELISA) quantification of osteoprotegerin concentrations in MG-63 conditioned media.....	76
3.11.2	Enzyme linked immunosorbent assay (ELISA) quantification of osteoprotegerin concentrations in MC3T3-E1 conditioned media.....	77
3.11.3	Enzyme linked immunosorbent assay (ELISA) quantification of free soluble RANKL (sRANKL) concentrations in MC3T3-E1 conditioned media.....	78
3.12	Detection of oestrogen receptors in MG-63 cells.....	78
3.13	Statistics.....	79

CHAPTER 4

Effects of Arachidonic acid, Oestrogen and Parathyroid Hormone on Prostaglandin E₂ (PGE₂) Production in MG-63 and MC3T3-E1 Osteoblast-like Cells..... 80

4.1	Introduction.....	80
4.2	Materials and methods.....	82
4.3	Results.....	87
4.3.1	Effects of cyclooxygenase blockers and arachidonic acid on prostaglandin E ₂ production in MG-63 cells.....	87

4.3.2	Indirect immunofluorescence staining for COX-1 and COX-2 in control and arachidonic acid-activated MG-63 cells.....	88
4.3.3	Effects of arachidonic acid, parathyroid hormone and oestrogen on prostaglandin E ₂ production in MG-63 and MC3T3-E1 cells.....	89
4.4	Discussion.....	92

CHAPTER 5

	Effects of Arachidonic Acid, Docosahexaenoic Acid, Prostaglandin E₂, Oestrogen and Parathyroid Hormone on Cell Proliferation and Morphology of MG-63 and MC3T3-E1 Osteoblast-like Cells.....	96
--	--	-----------

5.1	Introduction.....	96
5.2	Materials and methods.....	98
5.3	Results.....	103
5.3.1	Proliferation studies.....	103
5.3.2	Morphology study: Haematoxylin and eosin (H&E) cell staining.....	108
5.3.3	Hoechst 33342 and propidium iodide (HOE/PI) staining for detection of apoptosis.....	111
5.4	Discussion.....	114
5.4.1	Proliferation studies.....	114
5.4.2	Morphological studies.....	119
5.4.3	Conclusion.....	121

CHAPTER 6

	Effects of Arachidonic Acid and Docosahexaenoic Acid on Differentiation of and Mineralisation by MG-63 and MC3T3-E1 Osteoblast-like Cells.....	122
--	---	------------

6.1	Introduction.....	122
6.2	Materials and methods.....	125
6.3	Results.....	131
6.3.1	Alkaline phosphatase (ALP) activity as marker of early differentiation.....	131
6.3.2	Onset of mineralisation as marker of osteoblast maturation.....	132
6.3.3	Oil Red O staining versus alkaline phosphatase activity as markers of osteoblastic transdifferentiation into adipocytes.....	135
6.4	Discussion.....	142
6.4.1	Alkaline phosphatase (ALP) activity as marker of early differentiation.....	142
6.4.2	Onset of mineralisation as marker of osteoblast maturation.....	143
6.4.3	Oil Red O staining versus alkaline phosphatase activity as markers of osteoblast transdifferentiation into adipocytes.....	146
6.4.4	Conclusions.....	147

CHAPTER 7	
Effects of Arachidonic Acid, Docosahexaenoic Acid, Prostaglandin E₂, Oestrogen and Parathyroid Hormone on Osteoprotegerin (OPG) and RANKL Secretion by MG-63 and MC3T3-E1 Osteoblast-like Cells..	150
7.1	Introduction..... 150
7.2	Materials and methods..... 155
7.3	Results..... 160
7.3.1	Effects of arachidonic acid, prostaglandin E ₂ , and parathyroid hormone on osteoprotegerin secretion in MG-63 and MC3T3-E1 cells..... 160
7.3.2	Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion in MG-63 and MC3T3-E1 cells..... 162
7.3.3	Effects of arachidonic acid, docosahexaenoic acid, prostaglandin E ₂ , parathyroid hormone and oestrogen on RANKL secretion in MC3T3-E1 cells 164
7.3.4	Effects of arachidonic acid, prostaglandin E ₂ and parathyroid hormone on the osteoprotegerin/sRANKL ratio in MC3T3-E1 conditioned media..... 165
7.3.5	Detection of oestrogen receptors in MG-63 cells..... 166
7.4	Discussion..... 167
7.4.1	Effects of arachidonic acid, docosahexaenoic acid and prostaglandin E ₂ on osteoprotegerin secretion..... 168
7.4.2	Effects of arachidonic acid, docosahexaenoic acid and prostaglandin E ₂ on RANKL secretion and the osteoprotegerin/RANKL ratio in MC3T3-E1 osteoblasts..... 170
7.4.3	Effects of parathyroid hormone on osteoprotegerin and RANKL secretion and the osteoprotegerin/RANKL ratio..... 173
7.4.4	Effects of oestrogen on osteoprotegerin and RANKL secretion..... 174
7.4.5	Conclusions..... 175
CHAPTER 8	
Conclusions and Further Research.....	177
REFERENCES.....	183
ADDENDUM I.....	216
List of congresses where parts of the work were presented	
ADDENDUM II.....	217
List of abstracts and articles published from this work	

LIST OF FIGURES

Figure	Title of Figure	Page
2.1	A scanning electron micrograph of compact and trabecular bone.	7
2.2	The origins and locations of bone cells.	8
2.3	Origin and fate of osteoblasts.	9
2.4	Model of the osteoblast differentiation pathway.	10
2.5	Basic action of nuclear hormone receptors.	11
2.6	Differentiation of osteoclast progenitors into functionally active osteoclasts.	14
2.7	Interactions of osteoprotegerin, RANKL (OPGL) and RANK on the differentiation and activation of osteoclast precursors.	16
2.8	Illustration of the morphological events characteristic of apoptosis and oncosis.	18
2.9	Schematic diagram of the bone remodeling cycle.	20
2.10	Osteoclastic bone resorption.	22
2.11	Oestrogen regulation of osteoblasts, osteoclasts, and osteoclast differentiation via osteoprotegerin (OPG) and other growth factors and cytokines.	25
2.12	Regulation of osteoclast formation, function, and apoptosis by cytokines produced by bone marrow cells, osteoblasts, monocytes, T cells, and B cells.	32
2.13	The 'convergence hypothesis' for the regulation of osteoclast functions by cytokines.	33
2.14	The elongation and desaturation pathways for n-3 and n-6 fatty acids.	35
2.15	The synthesis of eicosanoids from polyunsaturated fatty acids.	37
2.16	Role of polyunsaturated fatty acids in signal transduction.	39
2.17	Schematic diagram of the putative roles of prostaglandin E ₂ in bone resorption.	60
2.18	Speculative illustration of the possible effects of polyunsaturated fatty acids on bone loss.	61
3.1	Growth curve and cell maintenance.	67
3.2	Correlation between crystal violet-derived absorbance and cell number.	68
3.3	Example of a standard curve for the calculation of the amount of prostraglandin E ₂ in cell culture samples using RIA.	69

3.4	Standard curve for the calculation of alkaline phosphatase (ALP) activity in cell cultures using colorimetric para-nitrophenol hydrolysis.	73
3.5	Principle of the osteoprotegerin ELISA assay.	76
3.6	Examples of standard curves for the calculation of the amount of osteoprotegerin in the conditioned media from MG-63 and MC3T3-E1 cells.	77
4.1	The prostaglandin pathway indicating the enzymes involved in the synthesis of prostaglandin E ₂ from its substrate arachidonic acid.	82
4.2	Effects of cyclooxygenase blockers and arachidonic acid on prostaglandin E ₂ production in MG-63 cells.	87
4.3	Immunofluorescent staining for COX-1 and COX-2 in control and arachidonic acid-activated MG-63 cells.	88
4.4	Effects of arachidonic acid and parathyroid hormone on prostaglandin E ₂ production in MG-63 cells.	89
4.5	Effects of arachidonic acid and oestrogen on prostaglandin E ₂ production in MG-63 cells.	90
4.6	Effects of arachidonic acid, parathyroid hormone and oestrogen on prostaglandin E ₂ production in MC3T3-E1 cells.	91
5.1	Effects of arachidonic acid on MG-63 and MC3T3-E1 cell proliferation.	103
5.2	Effects of docosahexaenoic acid on MG-63 and MC3T3-E1 cell proliferation.	104
5.3	Effects of prostaglandin E ₂ on MG-63 and MC3T3-E1 cell proliferation.	105
5.4	Effects of oestrogen on MG-63 and MC3T3-E1 cell proliferation.	106
5.5	Effects of parathyroid hormone on MG-63 and MC3T3-E1 cell proliferation.	107
5.6	Photomicrographs of haematoxylin and eosin (H&E) stained MG-63 cells after 48 hours polyunsaturated fatty acid exposure.	109
5.7	Photomicrographs of haematoxylin and eosin (H&E) stained MC3T3-E1 cells after 48 hours polyunsaturated fatty acid exposure.	110
5.8	Photomicrographs of MG-63 cells after Hoechst and propidium iodide (HOE/PI) fluorescent staining for detection of apoptosis.	112
5.9	Photomicrographs of MC3T3-E1 cells after Hoechst and propidium iodide (HOE/PI) fluorescent staining for detection of apoptosis.	113
6.1	Stages in osteoblast development.	123
6.2	Alkaline phosphatase activity of MG-63 cells after 48 hours of exposure to polyunsaturated fatty acid and hormones.	131
6.3	Alkaline phosphatase activity of MC3T3-E1 cells after 48 hours of exposure to polyunsaturated fatty acid and hormones.	132

6.4	Alkaline phosphatase activity of MG-63 cells after 14 days of exposure to polyunsaturated fatty acid and hormones.	133
6.5	Alkaline phosphatase activity of MC3T3-E1 cells after 14 days of exposure to polyunsaturated fatty acid and hormones.	134
6.6	Photomicrograph of mineralised nodules.	136
6.7	Alkaline phosphatase activity of MG-63 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.	137
6.8	Alkaline phosphatase activity of MC3T3-E1 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.	138
6.9	Quantification of Oil Red O staining of MG-63 cells after six days of exposure to arachidonic acid and docosahexaenoic acid.	139
6.10	Photomicrographs of Oil Red O staining in MG-63 and MC3T3-E1 cells.	141
7.1	The 'Convergence hypothesis' for the regulation of osteoclast functions by cytokines.	153
7.2	Effects of arachidonic acid, prostaglandin E ₂ , and parathyroid hormone on osteoprotegerin secretion by MG-63 cells.	160
7.3	Effects of arachidonic acid, prostaglandin E ₂ , and parathyroid hormone on osteoprotegerin secretion by MC3T3-E1 cells.	161
7.4	Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion by MG-63 cells.	162
7.5	Effects of docosahexaenoic acid and oestrogen on osteoprotegerin secretion by MC3T3-E1 cells.	163
7.6	Effects of arachidonic acid, prostaglandin E ₂ , parathyroid hormone and oestrogen on RANKL secretion by MC3T3-E1 cells.	164
7.7	Effects of arachidonic acid, prostaglandin E ₂ , and parathyroid hormone on the osteoprotegerin/sRANKL ratio in MC3T3-E1 cells.	165
7.8	Oestrogen receptor detection in MCF-7 human breast carcinoma cells and MG-63 human osteosarcoma cells.	166

LIST OF TABLES

Table	Title of Table	Page
3.1	Composition of maintenance culture media and freeze media used.	63
3.2	Preparation and storage conditions of compounds used.	66

LIST OF ABBREVIATIONS

AA	arachidonic acid (C20,5c,8c,11c,14c-20:4)[n-6]
ALA	α -linolenic acid (C18,9c,12c,15c-18:3)[n-3]
ALP	alkaline phosphatase
α -MEM	alpha modification of Eagle's minimal essential medium
ANOVA	analysis of variance
β -GP	β -glycerophosphate
BMP	bone morphogenetic protein
BMP-2	bone morphogenetic protein-2
BSA	bovine serum albumine
BSS	balanced salt solution
caspsases	cysteinyl aspartate-specific proteases
cAMP	cyclic AMP
Cbfa1	core binding factor α -1
cdks	cyclin-dependent kinases
CLA	conjugated linoleic acid
COX	cyclo-oxygenase
COX-1	cyclo-oxygenase-1
COX-2	cyclo-oxygenase-2
Col1a1	type I collagen
cPGES	cytosolic prostaglandin E synthase
cPLA ₂	cytosolic phospholipase A ₂
DAG	diacylglycerol
ddH ₂ O	deionised distilled water
DGLA	dihomo-gamma-linolenic acid (C20,8c,11c,14c-20:3) [n-6]
DHA	docosahexaenoic acid (C22,4c,7c,10c,13c,16c,19c-22:6)[n-3]
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
E2	oestrogen (17 β -estradiol)
EDTA	disodium ethylene diaminetetraacetate
EFA	essential fatty acids
EGTA	ethylene glycol-bis[beta-aminoethyl ether]N,N,N ₁ ,N ₁ -tetraacetate
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E ₂ receptor
EPA	eicosapentaenoic acid (C20,5c,8c,11c,14c,17c-20:5)[n-3]
ER	oestrogen receptor
Erk	extracellular signal-regulated kinase
FCS	fetal calf serum

FGFs	fibroblast growth factors
FITC	fluoroisothiocyanate
GLA	gamma linolenic acid (C18,6c,9c,12c-18:3)[n-6]
GPCRs	G protein-coupled receptors
H ⁺ -ATPase	proton ATPase
H&E	Haematoxylin and eosin
hBMSc	human bone marrow stroma cells
hFOB	human fetal osteoblastic cell line
HOE	Hoechst no 33342
HOE/PI	Hoechst no 33342 and propidium iodide
IGFs	insulin-like growth factors
IGF-1	insulin-like growth factor-1
IGFBPs	insulin-like growth factor binding proteins
IL	interleukin
Indo	indomethacin
JNK	c-jun N-terminal protein kinase
LA	linoleic acid (C18,9c,12c-18:2)[n-6]
LO	lipoxygenase
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MC3T3-E1	mouse calvaria osteoblast-like cell line
MCF-7	human breast carcinoma cell line
M-CSF	macrophage-colony stimulating factor
MEM	minimum essential medium with Earle's salts
MG-63	human osteoblast-like osteosarcoma-derived cells
mPGES	membrane-associated prostaglandin E synthase
mRNA	messenger RNA
MSCs	mesenchymal stem cells
n-3	omega-3. Family of polyenoic fatty acids with 3 or more cis-unsaturated centres separated by methylene groups and having first unsaturated center 3C from the methyl terminal.
n-6	omega-6. Family of polyenoic fatty acids with 2 or more cis-unsaturated centres separated by methylene groups and having first unsaturated center 6C from the methyl terminal.
N.D.	not detected
NFκβ	nuclear factor κβ
NSAIDS	nonsteroidal anti-inflammatory drugs
O.D.	optical density
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand (RANKL)
Osx	osterix

OVX	ovariectomised
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PGs	prostaglandins
PGE ₂	prostaglandin E ₂
PGG ₂	prostaglandin endoperoxide G ₂
PGH ₂	prostaglandin endoperoxide H ₂
PGHS-1	prostaglandin endoperoxide synthase-1
PGHS-2	prostaglandin endoperoxide synthase-2
PGI ₂	prostacyclin
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PLA ₂	phospholipase A ₂
p-NP	para-nitrophenol
p-NPP	para-nitrophenylphosphate
PPAR	peroxisome proliferator activated receptor
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
PUFA	polyunsaturated fatty acid
PUFAs	polyunsaturated fatty acids
RANK	receptor activator of nuclear factor- κ β
RANKL	receptor activator of nuclear factor- κ β -ligand
RIA	radioimmunoassay
rpm	revolutions per minute
RXR	retinoid X receptor
SDF-1	stromal cell-derived factor-1
sRANKL	soluble secreted RANKL
SREBP	sterol regulating element binding protein
TBS	tris-buffered saline
TGF- β	transforming growth factor- β
TMB	3',3',5',5' tetramethylbenzidine
TNF	tumor necrosis factor
TNF α	tumor necrosis factor- α
TRAF-6	TNF receptor-associated factor-6
TRAP	tartrate-resistant acid phosphatase
UV	ultra violet
v/v	volume per volume
vit D ₃	1,25-Dihydroxy vitamin D ₃ (calcitriol)
w/v	weight per volume