

Omega-3 fatty acid-rich fish oil supplementation prevents rosiglitazone-induced osteopenia in insulin resistant c57bl/6 mice and *in vitro* studies.

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Abstract

Rosiglitazone is an effective insulin-sensitizer, however associated with bone loss mainly due to increased bone resorption and bone marrow adiposity.

We investigated the effect of the co-administration of fish oil on rosiglitazone-induced bone loss in C57BL/6 mice and the mechanisms underlying potential preventive effect. Mice fed iso-caloric diet supplemented with fish oil exhibited significantly higher levels of bone density in different regions compared to the other groups. In the same cohort of mice, reduced activity of COX-2, enhanced activity of alkaline phosphatase, lower levels of cathepsin k, PPAR- γ and pro-inflammatory cytokines, and higher level of anti-inflammatory cytokines were observed.

Moreover, fish oil restored rosiglitazone-induced reduction of osteoblast differentiation and up-regulation of adipocyte differentiation in C3H10T1/2 cells and inhibited the up-regulation of osteoclast differentiation of RANKL-treated RAW264.7 cells.

We finally tested our hypothesis on human Mesenchymal Stromal Cells differentiated in osteocytes and adipocytes confirming the beneficial effect of docosahexaenoic acid during treatment with rosiglitazone, through the downregulation of adipogenic genes, such as adipin and FABP4 along the PPAR γ /FABP4 axis, and reducing the capability of osteocytes to switch toward adipogenesis.

Fish oil may prevent rosiglitazone-induced bone loss by inhibiting inflammation, osteoclastogenesis and adipogenesis and by enhancing osteogenesis in the bone microenvironment.

Introduction

Thiazolidinediones (TZDs, also known as glitazones) are a class of antidiabetic agents that act as insulin sensitizers. Rosiglitazone (RSG) and pioglitazone are the most commonly used anti-diabetic drugs of this class and they work as an insulin sensitizer, by binding to the peroxisome proliferator-activated receptors (PPAR)- γ in fat cells and making the cells more responsive to insulin.

Glitazones are recommended in the algorithm of the American College of Endocrinologists for the treatment of Type 2 Diabetes (T2D), as per the latest consensus statement in January 2020 ¹. They are the only antihyperglycemic agents able to reduce insulin resistance, in addition to potent A1C-lowering properties associated with low risk of hypoglycemia and durable glycemic effects ¹⁻³. Pioglitazone may confer cardiovascular diseases (CVD) benefits ^{2,4,5}, while RSG has a neutral effect on CVD risk ^{6,7}. The use of TZDs has been limited by the onset of side effects, such as weight gain, increased bone fracture risk in postmenopausal females and elderly males, and an elevated risk for chronic edema or heart failure ^{1,8-11}.

RSG (Avandia[®], GlaxoSmithKline) is a potent TZD insulin sensitizer that decreases hyperglycemia by reducing insulin resistance in patients with T2D ³. It is used as a stand-alone drug or in combination with metformin or glimepiride. Despite RSG's effectiveness at decreasing blood sugar in T2D, its use decreased dramatically as studies showed apparent associations with increased risks of heart attacks and death ¹². Although extensive controversies raised by the detection of side effects, especially increased risk of heart failure ⁸, the Food and Drug Administration (FDA) decided to keep the drug available in the US; from November 2011 until November 2013, Avandia[®] was sold only with a prescription from a certified doctor. In November 2013, the FDA lifted its earlier

restrictions on RSG after reviewing the results of the 2009 RECORD (Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of glycemia in Diabetes) clinical trial: a six-year, open-label randomized control study that did not show any significant effect of RSG on myocardial infarction, being also the risk of overall cardiovascular morbidity or mortality comparable to other standard glucose-lowering drugs ⁶.

Despite the controversies on the risk profile side effects, RSG still remains a drug of choice for many T2D patients because of its unique insulin-sensitizing capacity¹. Side effects variably associated with RSG include increased bone loss and adiposity, risk of myocardial infarction and other CVD^{13,14}, and decreased serum bone-specific alkaline phosphatase (ALP) in older diabetic patients¹⁵. The long-term follow up of the RECORD 2009 study showed that consistently with the main study, RSG is associated with an increased risk of peripheral bone fracture in women, and probably in men, however without an increase in potentially high-morbidity (hip, pelvis, femur, and spine) fractures¹⁶.

A review of fracture risk associated with glitazone therapy in preclinical studies demonstrated that activation of PPAR γ inhibits bone formation primarily by diverting Mesenchymal Stromal Cells (MSCs) to the adipocytic rather than to the osteogenic lineage and that glitazones may also increase bone resorption by stimulating osteoclasts¹⁷. There is evidence suggesting that inflammatory diseases can increase bone resorption and decrease bone formation, most commonly through their impact on both osteoclastogenesis and osteoblastogenesis processes¹⁸.

Considered that the safety of this powerful therapeutic agent is still under strict scrutiny, there is an imperative need to develop preventive strategies to reduce RSG-mediated bone loss in T2D patients. Both T2D and osteopenia are considered to be inflammatory disease^{19,20} and fish oil (FO) rich in omega-3 fatty acids (FAs) are natural anti-inflammatory compounds²¹⁻²³. FO has been shown to reduce the incidence of a wide range of degenerative disorders and medical problems, including CVD and sudden death in epidemiological and clinical trials^{24,25}. In 2004, FO was recognized and approved by the FDA for the treatment of hyperlipidemia (LOVAZA™ Reliant Pharmaceuticals, now, GSK Inc, USA). Potential beneficial actions of FO on the cardiovascular system^{24,25} and numerous non-cardiac conditions, including inflammatory and arthritic disorders²⁶, neurological /neuropsychiatric disorders, ophthalmic disorders, gynecological problems, cancer²⁷, autoimmune disease, and osteoporosis²³ have been investigated²⁸.

In previous studies, we have demonstrated that dietary supplementation with FO protects against ovariectomy-induced bone loss at the femur and lumbar vertebrae in mice²⁹. We hypothesize that this protection is due to n-3 polyunsaturated fatty acids (n-3 PUFAs), especially eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3), richly found in FO. In humans, elevated concentration of n-3 FAs was positively correlated with n-3 PUFA content and Bone Mineral Density (BMD)³⁰. Additionally, *in-vitro* studies using the preosteoblastic cell line MC3T3-E1 indicated that the production of the bone-formation markers, alkaline phosphatases (ALP) and osteocalcin, were higher after 48 h of treatment with EPA, compared with arachidonic acid (AA)³¹. Furthermore, we have confirmed that DHA is even more potent than EPA in inhibiting RANKL-stimulated osteoclast differentiation, activation, and function in the RAW 264.7 cell line³². These *in-vitro* and *in-vivo* animals and human studies have suggested that FO may attenuate inflammation and reduce RSG-mediated postmenopausal bone loss. However, whether FO can alleviate RSG-mediated increased bone loss has not been previously explored.

To evaluate the efficacy and elucidate potential mechanisms of FO in the prevention of RSG-mediated bone loss, we have conducted three separate sets of experiments:

- a. *In vitro*, we explored possible mechanisms by which the FO components, EPA and DHA, might reduce bone resorption – measured by RANKL-stimulated osteoclast differentiation in RAW264.7 cells and osteoclastic activity on sperm whale dentin slice, induced by parathyroid hormone-related protein (PTHrP) in young mouse bone marrow (BM) cells, with or without RSG. We also examined the osteogenesis and adipogenesis induced by bone morphogenetic protein-2 (BMP-2) in C3H10T1/2 cells, and osteogenesis stimulated by ascorbic acid and β -glycerophosphate in mouse BM stromal cells *in-vitro*.
- b. *In vivo*, we investigated the protective effects of FO against RSG-mediated bone loss in aging C57Bl/6J mice, a strain susceptible to obesity, hyperglycemia and insulin resistance when fed corn oil (CO)-based diet³³.
- c. Finally, as an assay of human readout, we tested the effect of DHA on human adipose tissue-derived MSCs (AD-MSCs) during differentiation towards osteocytes and adipocytes *in vitro*, showing how gene expression is modulated.

Results

In-vitro experiments

Omega-3 FAs attenuate RSG-mediated stimulation of adipogenesis, and reduction of osteoblast differentiation in C3H10T1/2 cells

C3H10T1/2, a cell line established from an early mouse embryo with a high degree of sensitivity to post-confluence inhibition³⁴, has been extensively used as an *in-vitro* model to examine mesenchymal differentiation into various phenotypic lineages by different inductive mediators³⁵. It has been demonstrated in multiple studies that, on BMP-2 stimulation, C3H10T1/2 cells undergo differentiation to an osteogenic phenotype^{36,37}. ALP is an important marker for osteoblast activity during early osteoblast differentiation. In this study, we exposed confluent C3H10T1/2 cells to rhBMP-2 in the presence of RSG with or without omega-3 FAs and determined the effect on osteoblast differentiation by ALP activity. Our results demonstrated that RSG significantly reduced the BMP-2-stimulated osteoblast differentiation and omega-3 FAs co-administration restored it (**Fig. 1**). We also determined the effect of RSG with or without omega-3 FAs on adipogenesis in C3H10T1/2 cells stimulated rhBMP-2. Our results showed that RSG induces the adipocyte differentiation in C3H10T1/2 cells and BMP-2-induced adipogenesis is significantly higher in RSG-treated cells (**Fig. 1**). However, omega-3 FAs co-administration could attenuate this effect (**Fig. 1**). Interestingly, the effect of DHA was stronger than EPA in reducing adipogenesis, indicating that omega-3 FA-rich FO has the potential to be an effective adjuvant dietary supplement with the RSG for T2D to attenuate RSG-mediated stimulation of adipogenesis and reduction of osteogenesis.

Effect of RSG with or without omega-3 FA on osteogenesis in BM stromal cells

We further tested the effect of RSG with or without omega-3 FA on osteogenesis in ascorbic acid and β -glycerophosphate treated BM stromal cells by measuring the levels of matrix calcification by alizarin red staining and osteoblastic activity by ALP activity. Our results showed that RSG significantly reduced the matrix calcification in BM stromal cells and omega-3 FAs co-administration restored it to as of control. Similarly, RSG significantly reduced the ALP activity and omega-3 FAs co-administration reinstated it (**Fig. 1S**).

Effect of RSG with or without omega-3 FAs on osteoclastogenesis in RAW 264.7 cells

Bone density depends on the delicate balance of three cell lineages: osteoblasts, adipocytes, and osteoclasts. Therefore, we determined the effect of RSG with or without omega-3 FAs on osteoclasts formation in RAW 264.7 cells stimulated with RANKL, by TRAP staining. Our results showed that RSG significantly enhanced the osteoclast differentiation in RAW 264.7 cells, and omega-3 FAs co-administration restored it to as of control (**Fig. 1S**). Interestingly, the effect of DHA was stronger than EPA in reducing osteoclastogenesis even significantly less than control (**Fig. 2**). This data supports our previously published study that DHA is a more potent inhibitor of osteoclast differentiation than EPA by suppressing cell fusion³². Whereas, in the present study, we found that RSG promoted cell fusion to make giant osteoclasts (**Fig. 2**).

Effect of RSG with or without omega-3 FAs on osteoclastic activity in BM cells

We further tested the effect of RSG with or without omega-3 FAs on osteoclastic activity on dentin slice using BM cells stimulated with PTHrP by measuring TRAP activity. Our results showed that omega-3 FAs co-administration attenuated RSG-mediated activation of osteoclastic activity of BM cells on dentin slice (**Fig. 2S**). As seen in other experiments, DHA had a better anti-osteoclastogenic effect when combined with RSG than that with EPA (**Fig. 2S**). These data indicate that omega-3 FAs rich FO has the potential to be an effective adjuvant dietary supplement, able to attenuate RSG-mediated stimulation of osteoclastogenesis, and thus to reduce bone resorption.

In vivo experiments

Body weight, food intake, and serum metabolites

All experimental groups were provided with an equivalent amount of daily calories using the LC standard rodent diet as the control; the different dietary treatments did not affect food intake in any group. However, body weight gain was ~ 33% higher in CO fed mice compared to a ~3% increase in the LC fed mice. This is consistent with the well-characterized obesity, insulin resistance, and T2D seen previously in these mice when fed a fat-supplemented diet³³. The RSG+CO, FO, and RSG+FO mice showed a ~10% decrease in body weight gain compared to that of the CO fed mice, even though there was no difference in food intake between these groups. Insulin resistance, which is strongly associated with obesity, is thought to be also caused by metabolic messengers, such as free FAs, produced by adipose tissue that inhibit insulin action on muscle³⁸. Furthermore, non-esterified fatty acid (NEFA) levels were significantly decreased in RSG+CO and RSG+FO compared to CO-fed mice. Increased fasting insulin and decreased glucose values in RSG+CO and RSG+FO fed mice compared to CO fed mice were observed (**Table 1**). Interestingly, the addition of RSG with FO did not alter the pharmacological activity in controlling hyperglycemia. This finding confirms the compatibility of RSG with FO and indicates the absence of a diet-drug interaction.

Prevention of RSG-mediated BMD loss by FO in C57BL/6 aging mice

BMD was measured at 13 months of age (baseline) and after 5 months of experimental diets (final value). Data were expressed as percent difference in BMD and analyzed from the endpoint value to the baseline value. Following 5 months of dietary supplementation with FO, aging RSG-treated female C57BL/6J mice displayed increases of 5-7% in BMD of the spine, distal femoral metaphysis (DFM), and proximal tibial metaphysis (PTM). These increases were in striking contrast (10-28% vs 5-10%) to the decline in BMD displayed by the RSG+CO mice, even greater than those observed in CO and LC mice. RSG+CO fed mice showed an accelerated loss in BMD compared to control CO and LC fed mice (**Fig. 3**). A notable reduction of BMD in all regions of bone was observed in RSG treated mice (**Fig. 3**). The RSG treated mice exhibited the lowest BMD in the PTM, femoral diaphysis, tibial

diaphysis, and the lumbar regions of the spine as compared to that of all the other groups. Interestingly, this reduction of BMD levels in all bone regions was restored when RSG was co-administered with FO (**Fig. 3**). These data support our *in vitro* findings that omega-3 FAs could attenuate the negative regulation of RSG on osteogenesis and adipogenesis, thus preventing RSG-mediated bone loss in animal models.

Ex-vivo

Effect of RSG with or without FO on bone markers of aging C57BL/6 mice.

After 5 months of experimental diets, BM cells were collected and stimulated with LPS. Quantitative real-time RT-PCR demonstrated that LPS-stimulated mRNA expression of both cathepsin k (a marker of bone resorption) and PPAR γ (a marker of fatty bone formation) was significantly increased in BM cells collected from RSG-treated animals and re-established in BM cells collected from RSG+FO group (**Fig. 4 A&B**). FO supplementation, either alone or in combination with RSG, decreases age-associated and RSG-mediated osteoclastogenic bone resorption, thus improving BMD (**Fig. 4A**). Furthermore, PPAR γ was significantly increased in RSG mice compared with RSG+FO mice (**Fig. 4B**). Cell lysates of whole BM cells from different experimental groups treated with LPS were also used to determine ALP (**Fig. 4C**) and COX-II activity (**Fig. 4D**). We noted that ALP activity was significantly decreased in RSG+CO mice, compared with CO and LC mice (**Fig. 4C**) and that FO supplementation among RSG-treated mice significantly blunted the RSG-related reductions in ALP activity in cultured BM. Levels of COX-II, which is known to stimulate osteoclastogenesis and bone resorption³⁹, were increased in the BM of CO and RSG+CO fed mice, whereas FO was shown to down-regulate COX-II expression, as a possible mechanism by which osteoclastogenesis and RSG-mediated bone resorption might be attenuated. Interestingly, we found a significant reduction of COX-II activity in LPS-treated BM cells from FO supplemented RSG mice, compared with CO and RSG+CO mice (**Fig. 4D**).

Effect of RSG with or without FO on inflammatory markers of aging C57BL/6 mice

Splenocytes and whole BM cells isolated from aging C57BL/6 mice on experimental diets were stimulated with LPS and analyzed for pro-inflammatory and pro-osteoclastogenic TNF- α and IL-6, and anti-inflammatory and anti-osteoclastogenic IL-10 cytokines. Interestingly, we found a significant decrease in IL-6 and TNF- α , and elevated IL-10 production by LPS-treated splenocytes (**Fig. 5A,B&C**) and BM cells (**Fig. 5D,E&F**) from FO and RSG+FO mice, compared with those from CO and RSG+CO mice. These results indicate that the reduction of pro-inflammatory cytokines by BM and splenocytes may indirectly prevent RSG-mediated bone loss by inhibiting bone-resorbing osteoclastogenesis in aging mice. Accordingly, increased levels of IL-10 production by splenocytes and BM from FO and RSG+FO mice, compared with those of RSG+CO mice, may have prevented RSG-mediated bone loss by suppressing bone resorption.

Human readout in vitro

AD-MSCs consistently (more than 50% in all conditions) differentiated in osteocytes; the differentiation rate for adipocytes was lower (5-9%), as expected for the assay. The ratios between differentiated, stained regions and total areas were calculated through the ImageJ software and no significant changes were identified between RSG and RSG+DHA, both in the adipo- and osteo-differentiation conditions (**Fig. 6**).

The analyses of the broad gene panel covering adipogenesis and early and late osteogenesis showed that the addition of DHA reduces the relative expression of adipsin ($p < 0.0001$), FABP4 ($p < 0.0001$), RUNX2 ($p = 0.0223$) and

ALPL ($p=0.0009$), with a not statistically significant trend for PPAR γ ($p=0.222$) in the osteogenic assay (**Fig. 7**). Adipsin, or complement factor D, is an adipocyte-specific gene. It functions as an adipokine, a cell-signaling protein secreted by adipocytes, which regulates insulin secretion in mice, and promotes lipid accumulation and adipocyte differentiation.

In the adipogenic setting, the increase of FABP4 ($p=0.0017$) and OPG ($p=0.0051$) reached statistical significance (**Fig. 3S**). FABP4 encodes the fatty acid-binding protein found in adipocytes.

Discussion

Evidence suggests that chronic inflammatory diseases, of almost any cause, are associated with bone loss which may be due to the direct effects of inflammation, poor nutrition, reduced lean-body mass, immobility¹⁸, and also to the effects of treatments for different medical conditions, such as T2D⁴⁰. These mechanisms are quite complex and closely interrelated but are ultimately mediated through effects on the bone remodeling cycle. T2D is an inflammatory disease and is associated with an increased risk of osteoporotic fractures, resulting in disabilities and increased mortality⁴¹. Moreover, drugs used in the treatment of T2D, specifically PPAR γ agonists, have also been reported to affect bone cell function and result in reduced BMD and increased risk of osteoporosis and fractures^{16,42}. TZDs, a class of antidiabetic agents that act as insulin sensitizers, bind PPAR γ *in vivo* with high affinity. PPAR γ regulates target gene transcription as a heterodimer with the retinoid X receptor. This heterodimeric complex is activated synergistically by TZDs⁴³. TZDs in general, and RSG in particular, enhance adipogenesis in stromal cells⁴⁴. Activation of PPAR γ stimulates adipogenesis and inhibits osteoblastogenesis *in vitro* in murine BM-derived clonal cell line⁴⁵, and *in vivo* in mice; these processes are in turn associated with the bone loss^{46,47}. Both osteocytes and adipocytes are generated from the same MSCs progenitors. TZDs inhibit osteoblast differentiation and stimulate adipocyte differentiation from MSC as a potential mechanism through which they exert their bone destructive effect^{48,49}. Using different animal models of osteoporosis, we demonstrated that FO rich in omega-3 FAs attenuates osteoporotic bone loss by down-regulating inflammatory mediators⁵⁰⁻⁵⁶. In this study, we demonstrated that components of FO, particularly DHA can revert RSG-induced adipogenesis in a murine C3H10T1/2 cell line. Previously, it has been shown that RSG induces PPAR γ expression and activity in primary BM stromal cells and C3H10T1/2 cells and that cyclic loading counteracts these effects⁵⁷. In this study, we found a similar effect with FO supplementation in counteracting RSG-induced PPAR γ expression in primary BM cells as well as RSG-induced adipogenesis in C3H10T1/2 cells, which prevented RSG-mediated bone loss in aging C57BL/6 mice. This is consistent with our earlier finding that feeding the FO diet in *fat1* transgenic mice prevented ovariectomy-induced bone loss by modulating the markers of inflammation and osteoclastogenic factors⁵⁸.

Generally, ALP is considered to be an early marker of osteoblast differentiation. As ALP is a byproduct of osteoblast activity, an increased level of ALP refers to active bone formation⁵⁹. It was previously shown that RSG-mediated ALP activity reduction in primary BM stromal cells and C3H10T1/2 cells can be counteracted by mechanical loading⁵⁷. Similarly, we showed here that FO components, particularly DHA, are able to effectively counterbalance the known RSG deleterious action on BM stromal cells, C3H10T1/2 cells and in an aging mouse model. Thus, FO supplementation prevents RSG-mediated activation of PPAR γ leading to adipocyte differentiation and down-regulation of ALP activity as described for mechanical loading. This could be one of the mechanisms through which FO supplementation restores RSG treatment-associated bone loss in aging C57BL/6 mice. In addition, obesity and insulin resistance are characterized by both excess adipose tissue and osteoporosis⁶⁰, two

diverse conditions that are nonetheless linked at the point of stem cell differentiation. Adipocyte and osteoblast formation occurs from a common progenitor, MSC^{61,62}, and signals that promote BM stromal cells differentiation toward one lineage may preclude the formation of the other. For example, when BM adiposity is increased in aging individuals, trabecular bone is essentially replaced by fat tissue⁶³. Increased adipocyte formation also leads to the secretion of pro-inflammatory cytokines, such as IL-6 and TNF- α , which are associated with osteoclastogenesis and enhanced bone-resorbing activity. In our study, we found that RSG stimulated the formation of giant osteoclast cells in the RAW264.7 line when stimulated with RANKL, possibly by inducing the fusion of TRAP-positive mononuclear cells. Earlier, we demonstrated that omega-3 FAs, particularly DHA inhibits the fusion of TRAP-positive mononuclear cells in RAW264.7 cells when stimulated with RANKL. Interestingly, the present study showed that omega-3 FAs treatment could attenuate RSG-mediated giant osteoclast formation in RAW264,7 cells. A significant reduction of TRAP activity of BM cells on dentin slice stimulated with PTHrp was also noted in RSG+FAs treated culture. This data reveals that FO supplementation may prevent the bone loss secondary to RSG treatment by reducing osteoclastic bone resorption.

A negative association between higher ratios of omega-6 (CO) to omega-3 (FO) FAs and BMD loss was also found in an earlier study on elderly men and women⁶⁴. Linoleic acid and AA are oxidized by lipoxygenases to form respectively 13-hydroxyoctadecadienoic acid (13-HODE) and 15-hydroxyeicosatetraenoic acid (15-HETE) respectively, which are known activators of PPAR γ ⁶⁵. In contrast, lipoxygenases convert EPA and DHA into 15-hydroxyeicosapentaenoic acid and 17-hydroxydocosahexanoic acid, which are not activators of PPAR γ . Thus, supplementation of FO may ameliorate BMD reduction by neutralizing PPAR γ activation by RSG in the BM microenvironment. Importantly, increased adiposity in BM might not only suppress osteoblastogenesis but might also promote bone resorption through the secretion of inflammatory cytokines capable of recruiting osteoclasts by BM adipocytes, much other fat cells elsewhere⁶⁶. We found that both the marker of bone resorption, cathepsin k⁶⁷ and marker of adiposity, PPAR γ ⁴⁵ mRNA levels are increased in LPS-stimulated BM cells collected from RSG-treated mice. However, FO co-administration restored both cathepsin k and PPAR γ mRNA levels. This data further supports the notion that FO supplementation may prevent RSG-mediated bone destruction by reducing BM adiposity and osteoclastic bone resorption.

It is well established that aging is associated with an increase in pro-inflammatory cytokines, such as IL-6 and TNF- α ^{68,69}. These cytokines are key regulators of osteoclastogenic activity and have been shown to increase bone resorption^{70,71}. Furthermore, they induce the expression of COX-II in osteoblastic and stromal cells, resulting in an increased production of PGE2, which is also an essential activator of osteoclastogenesis⁷². In this study, we found a lower activity of TNF- α and IL-6 in BM of in RSG+FO treated mice, which could in part explain the maintenance of higher BMD compared with RSG alone treated mice. Previous studies have shown that FO and omega-3 FAs decrease the expression and activity of these cytokines, both *in vivo* and *in vitro*^{29,51}, and we earlier demonstrated that immune cells and kidney tissues derived from FO-supplemented autoimmune disease-prone mice exhibited decreased levels of pro-inflammatory cytokines and lesser bone loss in these animals^{51,73}. Besides, IL-10, an anti-inflammatory cytokine, has been shown to inhibit osteoclast formation and bone resorption in rat and mouse systems⁷⁴. Interestingly, FO co-administration significantly increased the IL-10 levels as well as reduced COX-II expression to normal levels. It is known that increased levels of COX-II stimulates osteoclastogenesis and bone resorption³⁹.

In an effort to mimic *in vitro* the human setting in bone and adipose tissues during treatment with RSG and DHA, we also used the model of human MSCs, which are known to contribute to tissue repair and are capable of differentiating into osteoblasts and adipocytes⁷⁵. This set of experiments confirmed the beneficial effect of DHA during treatment with RSG, through the downregulation of adipogenic genes, such as adiponectin and FABP4 along the PPAR γ /FABP4 axis, which stands for a reduced capability of osteocytes to switch toward adipogenesis. On the other hand, there is a not concordant trend for osteogenic genes: RUNX2 expression is significantly increased while ALP is reduced in the cells treated with RSG and DHA compared to RSG alone.

Overall, the results on the adipogenic differentiation of MSCs are probably consistently biased by the low differentiation rate obtained on day 14, as per the manufacturer's instructions. We believe that more consistent and reliable results on the effect of RSG and DHA on adipocytes could be obtained with an analysis conducted at a higher differentiation threshold, given that the changes in expression required for adipose differentiation, in terms of expression of a new set of genes and silencing of others, is significantly greater compared to the osteogenic differentiation⁷⁶.

In addition to the already reported effect of FAs as complementary therapies with RSG to counteract dyslipidaemia and insulin resistance⁷⁷, our study suggests that dietary FO supplementation helps to improve RSG-mediated bone loss in aging mice and human osteo-differentiated MSCs by modulating inflammatory and osteoclastogenic factors. Further experiments with combinations of RSG and FO in osteoporotic animal models, supplemented with purified enriched EPA or DHA diet (with and without treadmill exercise), and *in vitro* human models are necessary to clarify the mechanisms involved in the protection of bone loss, and also to determine their specific differential potential.

Materials And Methods

All media and cell culture components, such as Histopaque, lipopolysaccharide (LPS), 4',6-diamidino-2-phenylindole, oil red O, L-ascorbic acid 2-phosphate, trypsin-EDTA reagent, BCIP/NBT buffered substrate tablets, RPMI-1640, α -Minimum Essential Medium (MEM) and PLTGold[®] Human Platelet Lysate were purchased from Sigma-Aldrich Inc. (USA). Additional supplies were purchased from these respective sources: fatty acids (EPA and DHA): Cayman Chemical Company, Ann Arbor, MI; Murine soluble RANKL: Pepro Tech Inc. Rocky Hill, NJ; tartrate-resistant acid phosphatase (TRAP) solution No. 387: Sigma Chemical Co., St. Louis, MO; BMP-2: Biovision, USA; RSG: Advance Scientific Inc. USA; penicillin and streptomycin: VWR; PTHrP: Bachem, USA; Qiashedder and RNeasy mini kits: Qiagen Inc. USA; One step cDNA synthesis kit for RT-PCR: Applied Biosystems, USA; GoTaq qPCR Master Mix: Promega, Madison, WI, USA; Bicinchoninic acid (BCA) protein assay kit: Pierce Inc, USA; Glucose (QuantiChrom, Hayward, CA), NEFA (Wako Pure Industries Ltd, Japan), ALP activity kit (ANASPEC, San Jose, CA) and insulin (Crystal Chem Inc. Research, Downers Grove, IL) were analyzed using commercially available kits following manufacturer's instructions. Tumor Necrosis Factor- α (TNF- α), Interleukin 6 (IL-6), and Interleukin 10 (IL-10) were measured by the Ready-set-go ELISA kit (eBioscience, Inc. San Diego, CA) according to the manufacturer's protocol. Differentiation kits for MSCs (StemPro[™] Adipogenesis StemPro[™] Osteogenesis Differentiation Kit) were bought from Gibco.

Samples from healthy volunteers are covered by Sidra Medicine IRB#1609004389. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Health Science Center at San Antonio, US. All animal experiments were performed in accordance with the guidelines and regulations approved by IACUC.

All methods were performed in accordance with the relevant guidelines and regulations.

The study was carried out in compliance with the ARRIVE guidelines.

In-vitro experiments

Cell cultures

C3H10T1/2 and RAW 264.7 cells were grown in a T75-flask (BD Falcon, USA) in a 5% CO₂ humidified atmosphere at 37°C. The C3H10T1/2 cells (clone 8 American Type Culture Collection, USA) and RAW 264.7 were cultured in α-MEM, supplemented with 10% Fetal Bovine serum (FBS) (HyClone Laboratories Inc. USA), L-glutamine and 1% antibiotics (50 U/mL penicillin and 50mg/mL streptomycin). After reaching a subconfluent state, C3H10T1/2 cells were trypsinized with 1x trypsin-EDTA and plated into 48 or 96-well culture plates based on experimental need; the medium was then changed every other day.

Cell proliferation assay

C3H10T1/2 cells were plated on 96-well cell culture plates (2x10⁴ cell/well) in 100mL of medium and grown for 24 hours at 37°C. Thereafter, RSG 1μM was added with or without EPA 10μM and/or DHA 10μM and the cells were incubated for another 72 hours. Cell proliferation was evaluated using an MTS proliferation assay kit (Promega Inc., USA)⁵⁵.

Osteoblast differentiation in C3H10T1/2 cells

C3H10T1/2 cells were plated at a density of 2x10³ cells per 500μL in a 48-well plate in α-MEM, supplemented with 10% FBS. After 24h incubation, the media were replaced with osteogenic media (supplemented with BMP-2 100 ng/mL) in the presence of RSG 1μM with or without EPA 10μM and/or DHA 10μM for 10 days. Media and factors were replaced every 3 days. Cells were then washed with cold phosphate-buffer saline (PBS) and fixed with 10% formalin. Cells were washed 2 more times with PBS and incubated in 500μL of ALP solution (1 BCIP/NBT tablet to 10 mL distilled water) (Sigma Aldrich, St. Louis, MO) at room temperature (RT) for 10–15 minutes or until the desired staining was achieved. Wells were then carefully rinsed with PBS/EDTA 20mM and the ALP-stained optical density (OD) intensity was analyzed by histomorphometry using the Metaview Image Analysis System (Olympus Inc. USA)⁷⁸.

ALP activity in C3H10T1/2 cells

C3H10T1/2 cells were plated at a density of 2x10³ cells per 500μL in a 48-well plate in α-MEM, supplemented with 10% FBS. After 24h incubation, the media were replaced with osteogenic media (supplemented with BMP-2 100ng/mL) in the presence of RSG 1μM with or without EPA 10μM and/or DHA 10μM for 7 days. Media and factors were replaced every 3 days. Cells were then washed with cold PBS and incubated with 1x lysis buffer at 4°C for 10 minutes with agitation. Cell suspensions were further centrifuged at 2500g for 10 minutes at 4°C. Supernatants were collected, and protein concentrations quantified with BCA assays to assure uniform protein contents among all samples. ALP activity was measured by using SensoLyte pNPP ALP Assay (AnaSpec, San Jose, CA, USA) according to the manufacturer's instructions. Fifty μg of protein extracts were transferred in duplicate to a 96-well plate; the pNPP reaction mixture was added to each well, and plates were incubated for 30

minutes before reactions were stopped by adding the stop solution. ALP activity in each sample was determined by measuring OD405 and compared to an ALP standard curve.

Adipogenesis in C3H10T1/2 cells

Adipogenesis was measured by histochemical techniques, as described by Wang *et al*³⁶. In *in-vitro* experiments, C3H10T1/2 cells were plated in a 48-well plate and cultured in a-MEM medium with 10% FBS supplemented with recombinant human (rh) BMP-2 (Biovision, USA) and cultured for 7 days in the presence of RSG 1 μ M with or without EPA 10 μ M and/or DHA 10 μ M. Cells were then subjected to oil red O histochemical staining with fast red AL salt as a substrate. The OD of each well was measured using Metaview Image Analysis System (Olympus Inc. USA)²⁰.

Osteoblast differentiation (Alizarin Red staining and ALP activity) in BM stromal cells

Stromal cells were generated from whole BM isolated from the femurs and tibias of 4-6 week old C57BL/6 female mice obtained from Jackson Laboratories (Bar Harbor, Maine). Cells were plated in a-MEM, supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, MO) in a T75 culture flask, and incubated at 37°C at 5% CO₂. After 24h of incubation, non-adherent cells were removed by replacing with fresh medium. Cells were allowed to grow to confluency and split at 1:2 ratios for a minimum of 6 weeks. Remaining viable cells generally belong to BM stromal type lineage. BM stromal cells were then plated at a density of 2x10⁴ cells per 100mL in a 96-well plate in a-MEM, supplemented with 10% FBS. After 24 hours of incubation, media were replaced with osteogenic media (supplemented with Ascorbic Acid 50 μ M and β -Glycerophosphate (Sigma Aldrich, St. Louis, MO) 10mM in the presence of RSG 1 μ M with or without EPA 10 μ M and/or DHA 10 μ M and incubated for an additional 7-10 days with media, factors and fatty acid changes every 2 days. Osteoblast differentiation was determined by two sets of experiments: Alizarin Red staining and ALP activity. For Alizarin Red staining, cells were washed with PBS and fixed with 10% formalin by incubating the plates at room RT for 15 min at the end of 10 days culture. Cells were then washed 3 times with PBS, avoiding to disturb the fragile monolayer of cells. After the final aspiration, Alizarin Red S Solution 50 μ L (Alizarin Red S 2g, distilled water 100mL, adjusted pH 4.1 to 4.3 using 0.5% ammonium hydroxide) (Sigma Aldrich, St. Louis, MO) was added for 20 minutes at RT. Wells were then carefully washed with distilled water and Alizarin Red S stained OD intensity was determined by histomorphometry using the Metaview Image Analysis System software⁷⁸. For ALP activity, at the end of 7 days culture, cells were washed with cold PBS and incubated with 1x lysis buffer at 4°C for 10 minutes with agitation. Cell suspensions were further centrifuged at 2500g for 10 minutes at 4°C. Supernatants were collected, and protein concentrations quantified with BCA assays to assure uniform protein contents among all samples. ALP activity was measured by using Sensolyte pNPP ALP Assay (AnaSpec, San Jose, CA, USA) according to the manufacturer's instructions. Fifty μ g of protein extracts were transferred in duplicate to a 96-well plate; the pNPP reaction mixture was added to each well, and plates were incubated for 30 minutes before reactions were stopped by adding the stop solution. ALP activity in each sample was determined by measuring OD405 and compared to an ALP standard curve.

Osteoclast differentiation in RAW264.7 cells

RAW264.7 cells were suspended in a-MEM containing FBS 10% and plated at a concentration of 2x10⁴ cells/well into a 48-well tissue culture plate in the presence of RANKL 50 ng/mL with or without RSG 1 μ M, with or without EPA 10 μ M and/or DHA 10 μ M. The medium, factors, and drugs were replaced after 3 days. After 5 days of culture, the cells were fixed and stained for TRAP using TRAP staining kit according to the manufacturer's instruction.

TRAP-positive cells with more than three nuclei were counted as TRAP-positive multinucleated cells (MNCs) and as osteoclast-like cells³².

TRAP activity of mouse BM cells on dentin slice

Sperm whale dentin slices were prepared as described earlier⁷⁹. Slices of sperm whale dentin were prepared using a Buehler low-speed diamond bone saw (Buehler, Lake Bluff, IL, USA) followed by sonication (15 minutes) in several changes of distilled water. Slices were polished between two glass plates, transferred to Petri dishes, and sterilized under UV light for 1 day. Slices were soaked in 100% ethanol, allowed to air dry, and rinsed twice with sterile saline solution. Before all experiments, slices were incubated in α -MEM supplemented with FBS 10% and penicillin-streptomycin solution 1% for at least 24h before use.

Four-week-old C57Bl/6J female mice were purchased from Jackson Laboratories (Bar Harbor, Maine 04609 USA). Whole BM cells were collected aseptically from the femora and tibiae, and plated in α -MEM, supplemented with FBS 10% and penicillin-streptomycin solution 1% in a T75 culture flask, and incubated at 37°C at 5% CO₂. After 4 hours of culture, non-adherent cells (osteoclast precursor cells) were collected, 1x10⁶ cells/500 μ L/well were gently plated on the dentine slices placed on 48-well tissue plate and cultured in the presence or absence of 40 ng/mL synthetic human PTHrP for 7 days at 37°C in a humidified 95:5 atmosphere of air/CO₂ (mol%) with or without RSG 1 μ M, with or without EPA 10 μ M and/or DHA 10 μ M as described earlier⁸⁰. The medium, factors, and drugs were replaced once in 3 days. A minimum of 3-4 slices was used per treatment.

For TRAP activity measurement on dentin slices, the medium was removed at the end of culture, and then cell monolayer was gently washed twice with PBS. The cells were then lysed with 200 μ L of 0.2% Triton X-100. TRAP activity in cell lysate was determined using the TRAP solution No. 387 kit. An aliquot of cell lysate was added to 150 μ L TRAP solution and was incubated at 37°C for 30 minutes. The absorbance was then measured at 570 nm using a microplate reader³².

In-vivo experiments

Mice strain

Ten-month-old female C57Bl/6J mice, weighing 23-25g, were purchased from Jackson Laboratories (Bar Harbor, Maine 04609 USA) and fed a standard diet (Harlan Teklad LM-485) for three months. Weight matched animals were then divided into five groups (n=10 per group) and housed in a standard controlled animal care facility. The animals were maintained in a temperature-controlled room (22-25°C, 45% humidity) on a 12:12 hours dark-light cycle. The mice were fed an American Institute of Nutrition diet (AIN93) supplemented with fat, with 10% of total calories coming from either CO or CO plus FO, in a 50/30/20 ratio of CO, EPA, and DHA, respectively. RSG was administered to half animals in each diet group (RSG+CO and RSG+CO+FO). All animals were fed *ad libitum* for 5 additional months. A fifth experimental group, fed a standard rodent lab chow (LC) diet, was also maintained throughout the experiment. Body weights and food intake of mice were measured weekly.

FO dietary supplement regime with RSG in C57Bl/6J aging mice

Five different dietary regimes were as follow: I) 10%CO; II) 5%CO and 5%FO; III) 10%CO plus RSG (RSG+CO); IV) 5%FO+5%CO plus RSG (RSG+FO) and V) standard rodent LC pelleted diet. The composition of the semi-purified AIN93 diet is presented in **Table 1S**. Groups I–IV were fed their experimental diets, produced by an in-house diet

preparation facility, for 5 months. RSG-treated animals (groups III and IV) were fed 5g of food per day, supplemented with RSG maleate at a concentration of 0.14 mg/g. Non-RSG-treated mice (groups I, II, and V) were fed the same quantity (5g/day) of CO diet, FO diet, or LC, respectively, similar to those used previously⁸¹. The mice were provided with fresh food every day in the afternoon (between 1:00-2:00 pm). The remaining food was removed daily to prevent rancidity. Diets were prepared each week, purged with nitrogen gas, frozen in daily portions, and sealed in polyethylene bags to minimize the oxidation of the fatty acids.

BMD measurements

BMD was determined using the Piximus instrument and software version 2.1 (GE Lunar, Madison, WI). Mice were anesthetized and scanned at 13 months of age, before the onset of the experimental diet, and then before sacrifice, at age 18 months. Total body BMD (g/cm²), excluding the head region, was obtained from each scan, and the percent change in BMD was determined as previously described²⁹.

Isolation of whole BM cells from aging C57BL/6 mice with experimental diets and determination of bone markers

After 5 months of experimental diets, whole BM cells were aseptically isolated at the time of sacrifice as described previously⁵¹. Cells were counted and viability was determined by trypan blue exclusion method. Cells (10x10⁶/well) were plated in 6-well plates and treated with bacterial LPS at a concentration of 5.0 µg/mL for 24h at 37°C, in a humidified atmosphere of air/CO₂ in a ratio of 95:5 (mol %). After 24h, cells and culture medium were collected together and centrifuged at 2000 rpm for 5 minutes. The cell pellets and culture supernatants were stored separately at -80°C. Cell pellets were used for gene expression, ALP (AnaSpec kit) and COX-II activity assays, while culture supernatants were used to analyze the levels of TNF-α, IL-6, and IL-10 cytokines.

Splenocyte preparation and culture

Spleens were aseptically removed from diet-fed mice at the time of sacrifice and placed in 5mL of RPMI 1640 medium supplemented with HEPES 25 mmol/L, glutamine 2 mmol/L, penicillin 100,000 U/L and streptomycin 100 mg/L. Single-cell suspensions were made by teasing spleens between frosted ends of two sterile glass slides. After 5-minute centrifugation at 100g to separate cells from debris, the cells were washed twice in RPMI medium. Splenic lymphocytes were isolated by layering over Histopaque, centrifuging at 1000 rpm for 15 minutes at 22°C, and then washing twice in RPMI 1640 complete medium. Cells were plated in 6-well plates (10x10⁶ cells/well) and viability was determined by the trypan blue exclusion method; bacterial LPS was added at a concentration of 5.0µg/mL for 24h at 37°C in a humidified 95:5 atmosphere of air/CO₂ (mol%). After 24h, the culture medium was collected and analyzed for TNF-α, IL-6, and IL-10 by standard ELISA techniques⁵³.

Cytokines measurement in the conditioned medium of cultured splenocytes and BM cells

TNF-α, IL-6, and IL-10 were measured in conditioned media by Ready-set-go ELISA kit (eBioscience, Inc. San Diego, CA) according to manufacturer's protocol⁵³.

RNA extraction and real-time RT-PCR

Real-time RT-PCR was used to determine the mRNA levels of cathepsin k and PPARγ. RNA was extracted from LPS-stimulated BM cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration of RNA was determined using NanoDrop™ (Thermo Scientific, Wilmington, DE, USA). Real-time

RT-PCR was then carried out using TaqMan[®] RNA-to-C_T 1-step kit (Applied Biosystems, Foster City, CA) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using fluorescent TaqMan system. Real-time quantitative RT-PCR was performed for each of the following genes using ready-to-use primer and probe sets developed by Applied Biosystems (TaqMan Gene Expression Assays): cathepsin k (*Ctsk*, Mm00484036_m1), PPAR γ (*ppary*, Mm01184321_m1) and Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1) as an endogenous control. mRNA Ct values for these genes were normalized to the house-keeping gene GAPDH and expressed as relative increase or decrease, compared with the CO group^{82,83}.

ALP quantification in BM cells

ALP activity was measured by using SensoLyte pNPP ALP Assay kit in 50 μ g protein extracts of 24h- cultured BM cells from experimental-diet and LC-fed mice, as described in *in-vitro* experiments.

Cyclo-oxygenase-II (COX-II) activation assay

100 μ g of cytosolic protein of 24h-LPS-treated BM cells from aging C57BL/6 mice with experimental diets were analyzed for COX-II activity using CAYMAN COX Activity Assay Kit according to the manufacturer's instruction⁵³.

Human readout in vitro

AD-MSC culture and differentiation

Human MSCs were isolated from discarded lipoaspirate of 2 healthy volunteers (Sidra Medicine IRB#1609004389). Automated and clinical-grade fat digestion was performed through the Celution[®] 800/CRS System (Cytori Therapeutics Inc., San Diego, USA). The isolated cells were seeded and cultured in flasks for expansion (2x10⁶ cells in T75 flask) with 5% PLTGold[®] Human Platelet Lysate (Sigma-Aldrich), low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich), 1% 10,000 U/mL penicillin and 10 mg/mL streptomycin (VWR), at 37°C in a humidified 95:5 atmosphere of air/CO₂ (mol%). The AD-MSCs were adherent to plastic, displayed fibroblastoid morphology and were compliant with the International Society of Cell Therapy criteria for MSCs⁸⁴. AD-MSCs were then cultured in adipo- (StemPro[™] Adipogenesis Differentiation Kit (Gibco)) and osteo- (StemPro[™] Osteogenesis Differentiation Kit (Gibco)) differentiating media, for 14 and 21 days respectively, as per manufacturer's instructions, with the following conditions: AD-MSCs (control), AD-MSCs+RSG 1 μ M (Cayman Chemical Company), AD-MSCs+DHA 10 μ M (Cayman Chemical Company), AD-MSCs+RSG+DHA. Each condition was run in duplicate for RNA extraction and staining.

AD-MSCs Staining

AD-MSCs were fixed with 4% Paraformaldehyde for 30 minutes and washed with water. Adipo-cultures were treated with 60% isopropanol for 5 minutes, stained with Oil Red O (Sigma) for 45 minutes, washed, and dried at RT. Osteo-cultures were directly stained with Alizarin-Red Staining Solution (Merck Millipore) for 45 minutes, gently washed, and finally dried at RT.

The plates were observed and photographed at an inverted microscope at 4x, 10x and 20x and images were analyzed with the ImageJ software.

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from differentiated AD-MSCs using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was DNase treated followed by first-strand cDNA using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen). Semi-quantitative analysis of relative gene expressions was performed on the QuantStudio™ 12K Flex System (Applied Biosystems, Carlsbad, CA) using GoTaq qPCR Master Mix (Promega, Madison, WI, USA). Primers were synthesized by Integrated DNA Technologies (Coralville, CA), sequences are listed in **Table 2S**. All primer pairs were validated by demonstrating high amplification efficiency and consistent single peak melt curve. The relative gene expression was normalized to β -Actin (endogenous control) and calculated using the $2^{-\Delta\Delta C_t}$ method setting the values of control as one.

Statistical analysis

Results are expressed as mean \pm SE. Data were statistically analyzed using one-way ANOVA; $P < 0.05$ was considered statistically significant. Newman–Keuls and Dunnett's multiple-comparison tests were used to test the significance of differences among group means.

Declarations

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Table 1

Table 1. Body weight and serum metabolites in C57Bl/6J aging mice after five months' dietary supplementation with CO, FO and/or RSG, respectively, compared with LC controls.

Values in parentheses are the differences between the pre- and post-supplementation values. Values with different letters, and sign indicates that values are significantly different by ANOVA, followed by Newman-Keuls multiple comparison post hoc test ($P < 0.05$).

Parameters	I CO	II FO	III RSG+CO	IV RSG+FO	V LC	
Body weights (n=15)						
Body weight(g)	Pre	23.7±0.94	23.2±0.50	23.2±1.12	23.3±0.72	23.5±0.75
	Post	31.7±1.2(33) ^a	28.5±1.21(23) ^b	28.9±0.87(24) ^b	28.7±0.8(24) ^b	24.2±0.81(3) ^b
Serum metabolites (n=10)						
Glucose (mg/dl)	194.1±7.2	157.2±4.2	129.6±4.3*	121.0±6.3*	80.12±4.2	
Insulin (ng/mL)	0.32±0.04	0.42±0.05	0.68±0.1*	0.72±0.1*	0.10±0.04	
NEFA (mmol/L)	1.27±0.03	0.90±0.02	0.87±0.05*	0.76±0.05*	1.06±0.12	

Figures

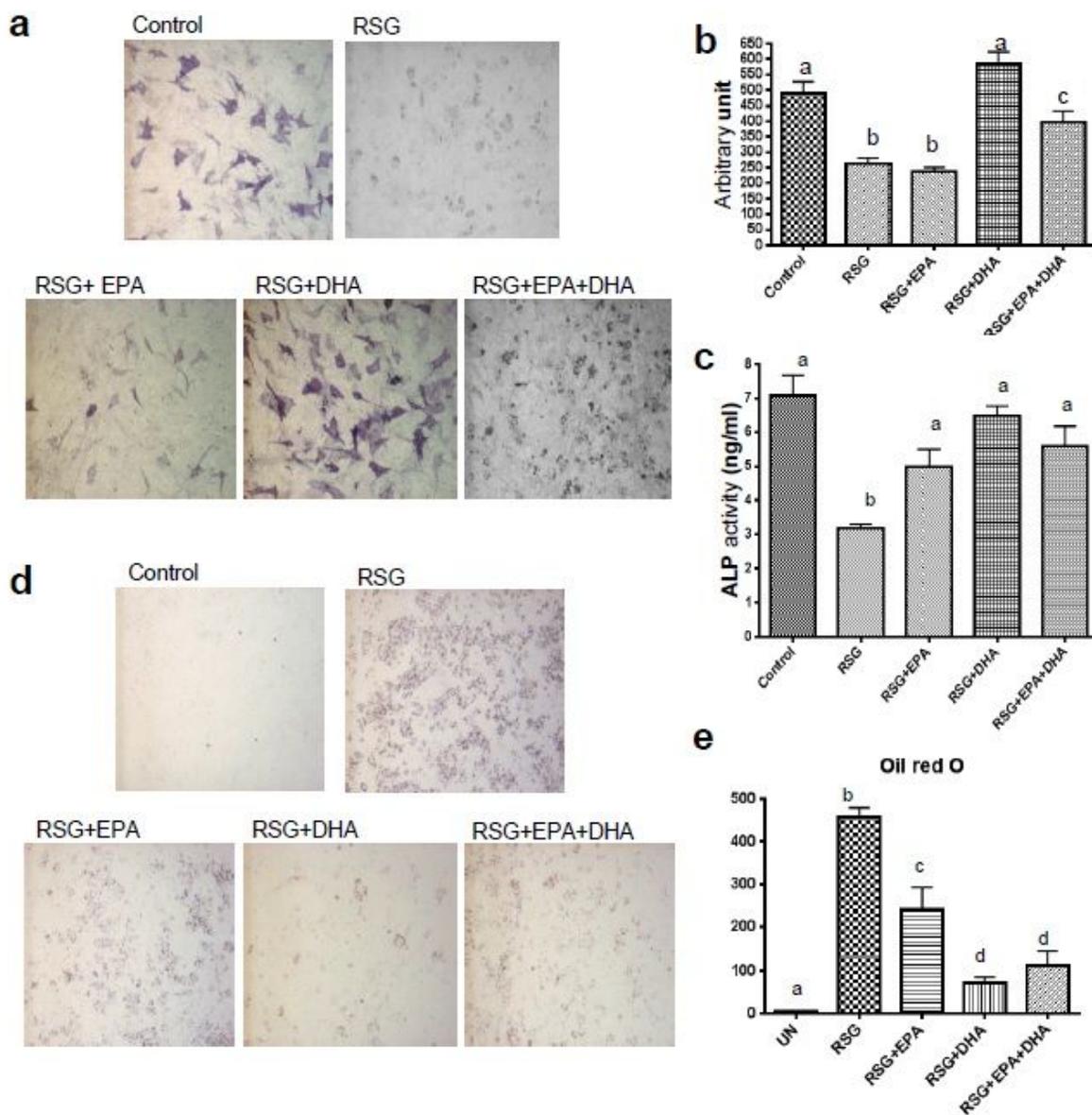


Figure 1

Effect of RSG with or without omega-3 FAs on osteoblastogenesis and adipogenesis in C3H10T1/2 cells

C3H10T1/2 cells were plated in a 48-well plate and cultured in α -MEM medium with 10% FBS supplemented with recombinant human (rh) BMP-2 (Biovision, USA) and cultured for 7 days in the presence of RSG 1 μ M with or without EPA 10 μ M and/or DHA 10 μ M. Representative photographs (20x) of C3H10T1/2 cells stained for alkaline phosphatase (elongated cells) (A). Optical density measured by Metaview Image Analysis (Olympus Inc. USA) in RSG treated cells with and without FO (EPA and DHA) in the presence of BMP-2 (B). Cells were lysed with lysis buffer and quantified for protein content. ALP activity was measured by using SensoLyte pNPP ALP Assay by mixing cell lysates with pNPP reaction mixture and incubated for 30 minutes. ALP activity in each sample was determined by measuring OD405 and compared to an ALP standard curve (C). Cells were then stained with Oil red O using fast red AL salt as a substrate. Representative photographs (20x) of C3H10T1/2 cells stained for Oil red O (round cells) (D). Optical density measured by Metaview Image Analysis (Olympus Inc. USA) in RSG-treated cells with and without FO (EPA and DHA) supplementation (E). Each bar represents the mean \pm SEM of 6 wells per group. Value with different superscripts is significantly different at $p < 0.05$ by Newman-Keuls' one way ANOVA with multiple comparison tests.

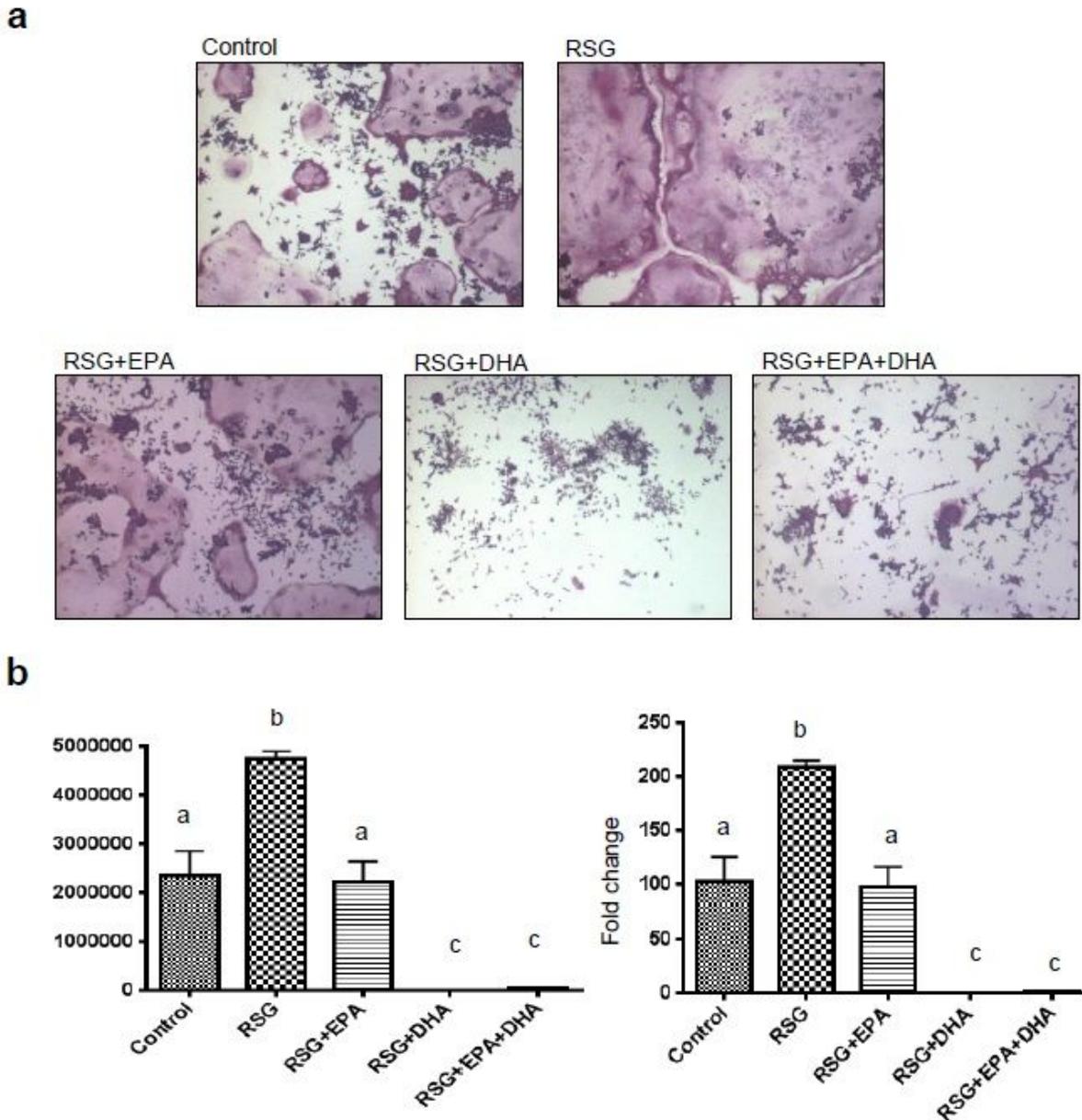


Figure 2

Effect of RSG with or without omega-3 FAs on osteoclastogenesis in RAW 264.7 cells RAW 264.7 cells were cultured with RANKL 50ng/mL in the presence of RSG 1 μ M with or without EPA 10 μ M and/or DHA 10 μ M for 5 days. At the end of cultures, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). The number of TRAP-positive multinucleated cells (MNCs) (more than 3 nuclei) was counted as osteoclast-like cells in each well. Representative photographs (20x) of TRAP-positive MNCs (A). Optical Density (OD) intensity was determined by histomorphometry using the Metaview Image Analysis System software for the TRAP-positive area (B). Each bar represents the mean \pm SEM of two independent quadruplicate cultures. Value with different superscripts is significantly different at P<0.05 by Newman Keuls one way ANOVA with multiple comparison test.

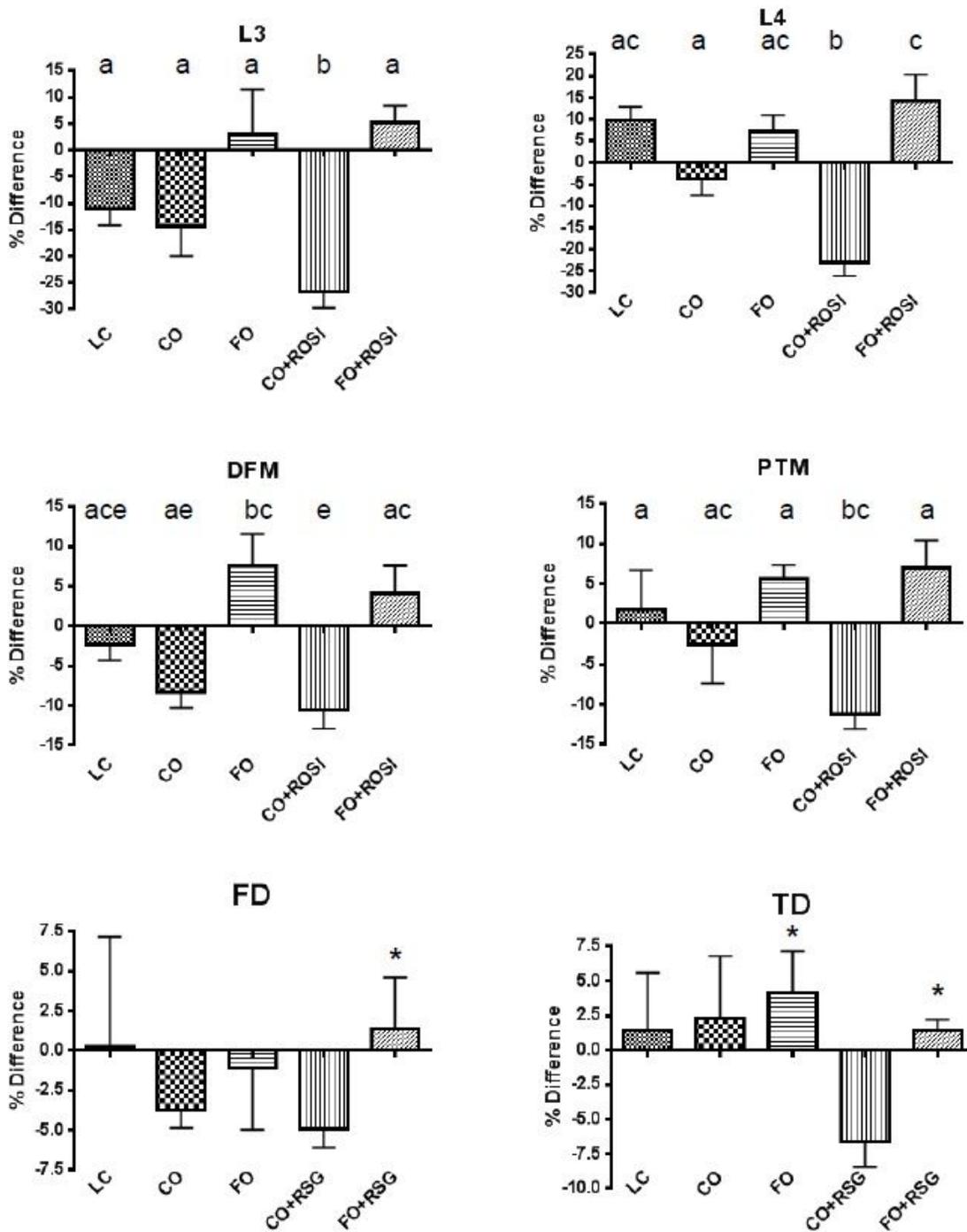


Figure 3

Effect of RSG with or without FO on bone mineral density (BMD) of aging C57BL/6 mice Thirteen months old C57BL/6 mice (n=10) were fed with CO, RSG+CO, FO, RSG+FO, and LC for 5 months. Region-specific BMD was measured by dual-energy x-ray absorptiometry (DEXA) for lumbar vertebra 3 (L3), lumbar vertebra 4 (L4), distal femoral metaphysis (DFM), proximal tibial metaphysis (PTM), femoral diaphysis (FD) and tibial diaphysis (TD). A DEXA scan was performed at 13 months of age just before starting the experimental diets to determine the baseline BMD value. Thirteen-months-old C57BL/6 mice were fed experimental diets for 5 months and then another DEXA scan was performed to determine the endpoint BMD value. The percent difference in BMD value was analyzed from the endpoint value to the baseline value. Each value represents the mean \pm SEM. n = 8–10 mice per

group. Values with different letters are significantly different by one way ANOVA followed by Newman-Keuls multiple comparison post hoc tests ($P < 0.05$). CO, corn oil; RSG, rosiglitazone; FO, fish oil; LC, lab chow.

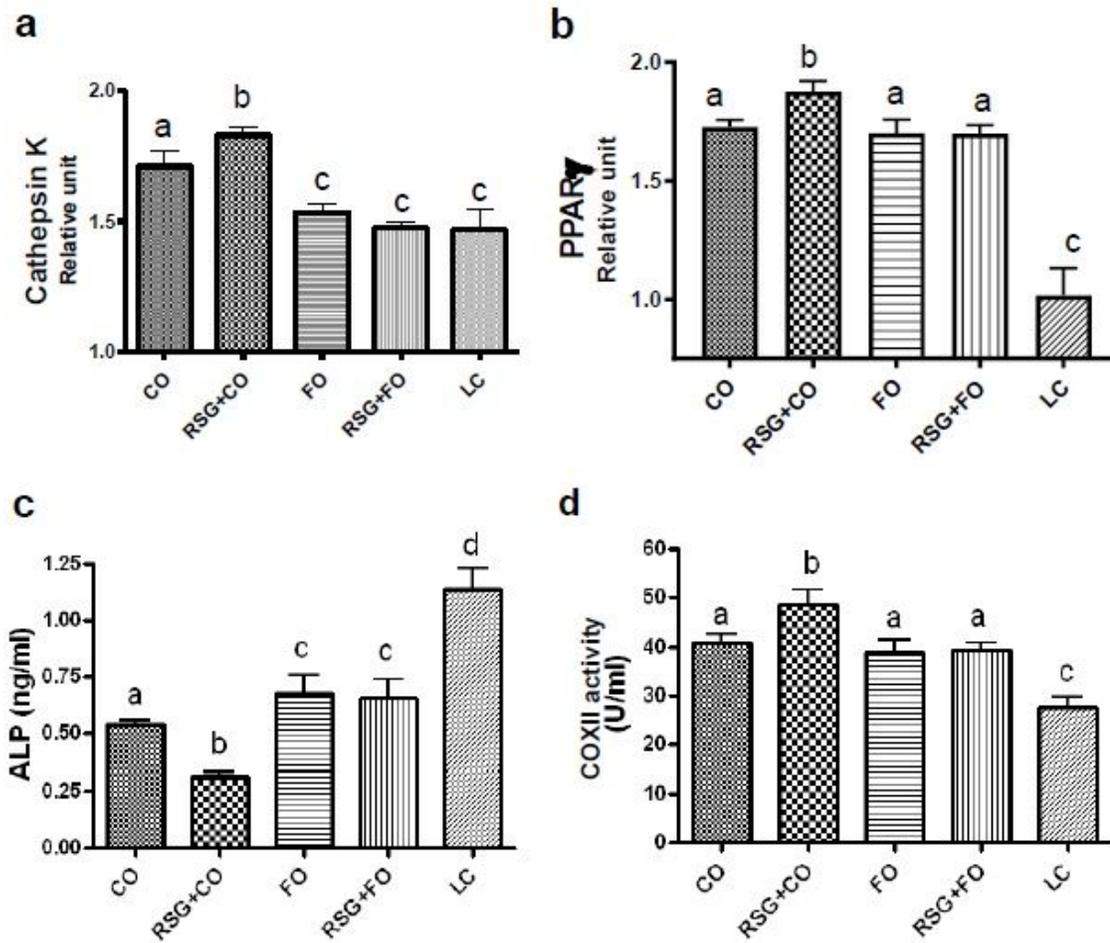


Figure 4

Effect of RSG with or without FO on bone markers of aging C57BL/6 mice At sacrifice whole bone marrow (BM) cells were collected from aging C57BL/6J mice (n=6) of each experimental diet group: CO, RSG+CO, FO, RSG+FO, or a standard lab chow control diet (LC) fed for 5 months. BM cells were treated with LPS for 24h and RNA was prepared from cell pellets; gene expression of cathepsin k (A) and PPAR γ (B) was determined by real-time RT-PCR. Cell lysates of whole BM cells from different experimental groups treated with LPS were also used to determine Alkaline phosphatase activity (C) and COX-II activity (D). Values with different letters are significantly different by one way ANOVA followed by Newman-Keuls multiple comparison post hoc tests ($P < 0.05$). CO, corn oil; RSG, rosiglitazone; FO, fish oil; LC, lab chow.

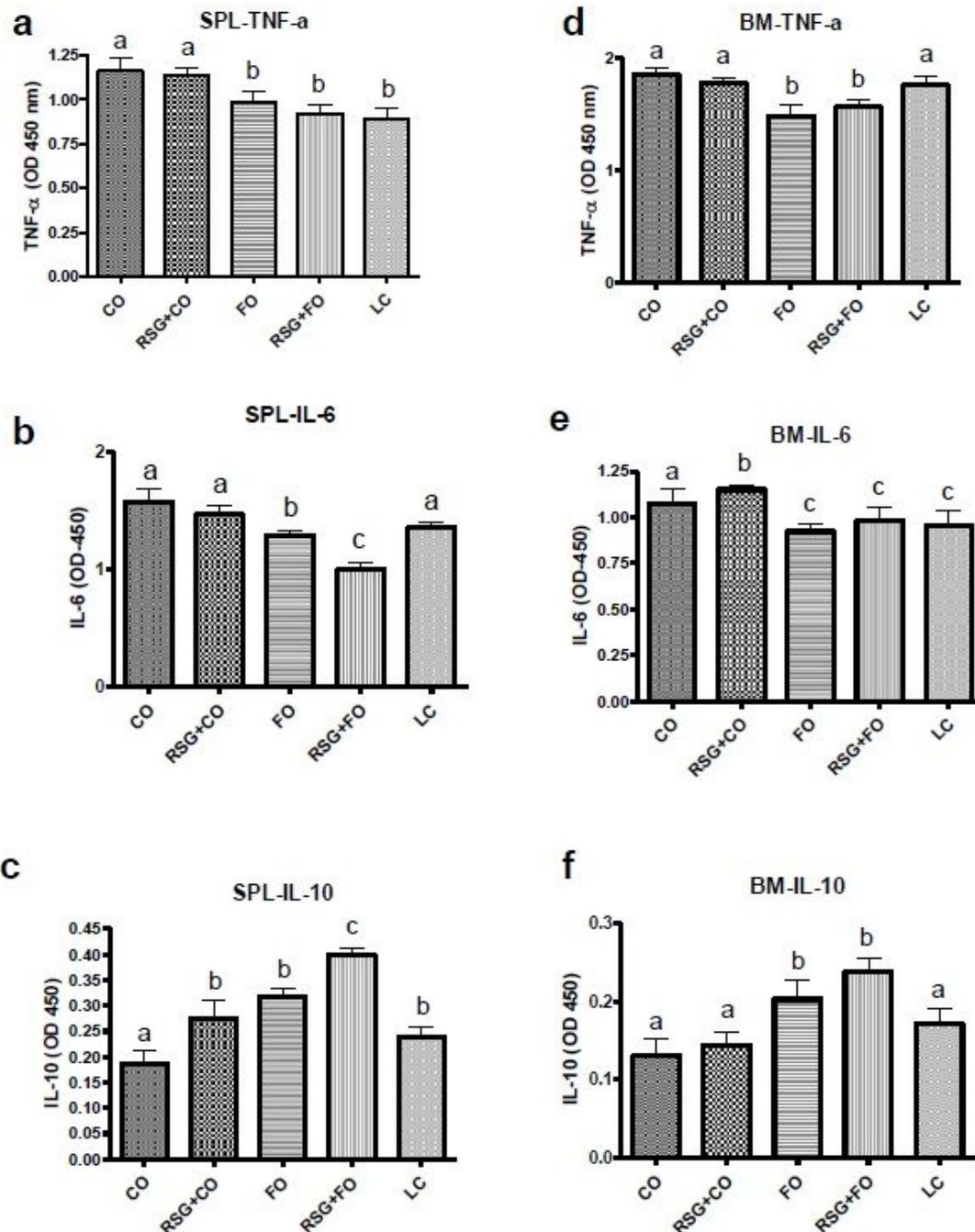


Figure 5

Effect of RSG with or without FO on inflammatory markers of aging C57BL/6 mice Splenocytes and whole bone marrow (BM) cells were isolated from aging C57BL/6 mice on experimental diets: CO, RSG+CO, FO, RSG+FO, and LC for 5 months. Splenocytes (A, B, C) and BM cells (D, E, F) were cultured separately and treated with LPS for 24 hours and culture supernatants were collected and analyzed for pro-inflammatory TNF- α , and IL-6 and anti-inflammatory IL-10 cytokines. n=5 mice per group. Values with different letters are significantly different by one way ANOVA followed by Newman-Keuls multiple comparison post hoc tests (P<0.05). CO, corn oil; RSG, rosiglitazone; FO, fish oil; LC, lab chow.

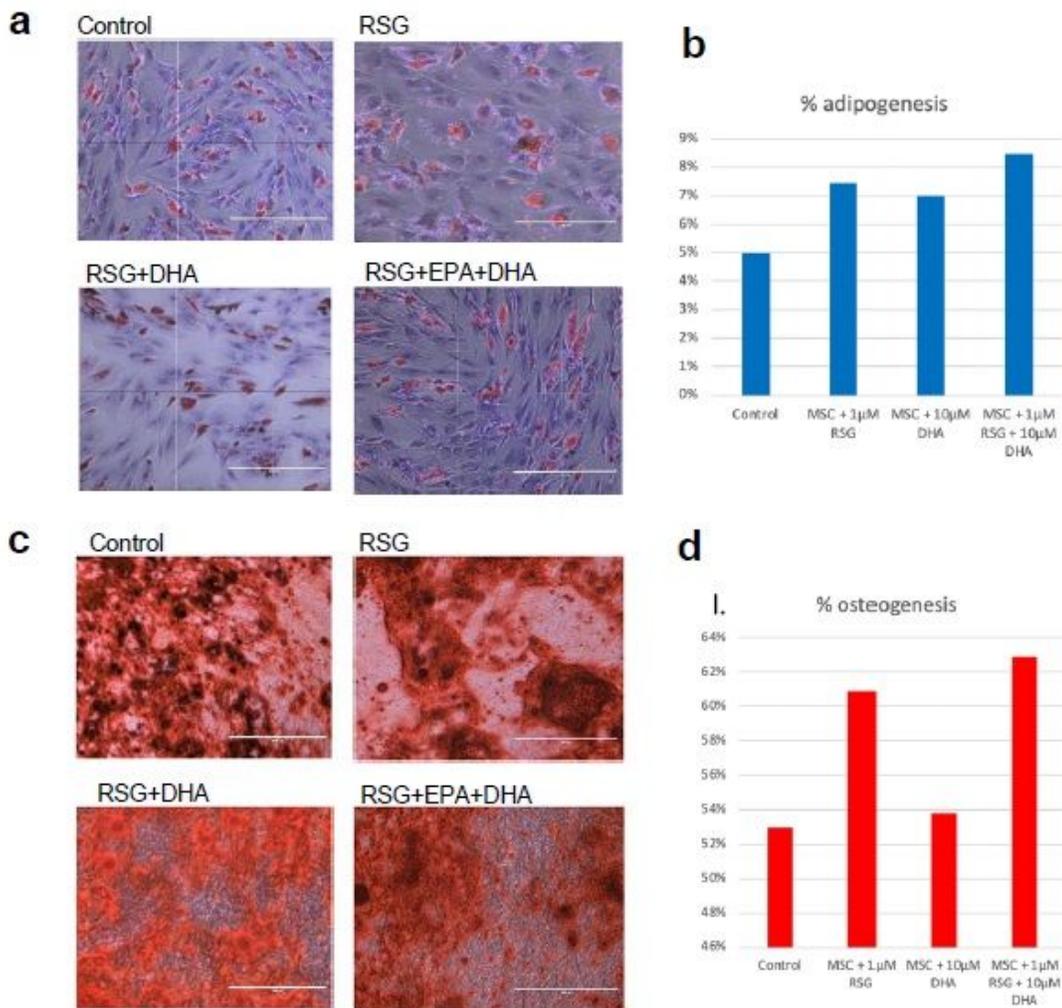


Figure 6

Effect of RSG with or without FO on adipo- and osteo-differentiation of human Mesenchymal Stromal Cells (MSCs). Adipose derived-MSCs were differentiated in adipogenic medium with the presence or absence of RSG, DHA and combination of RSG and DHA (a, b, c, d). The plates were photographed at an inverted microscope at 4x, 10x and 20x and images analyzed with the ImageJ software. The percentage of adipo-differentiated MSCs at the different conditions was calculated [ns- P value non-significant] (e); Adipose-derived -MSC were differentiated in osteogenic medium with the presence or absence of RSG, DHA and combination of RSG and DHA (f, g, h, i). The plates were photographed at an inverted microscope at 4x, 10x and 20x and images analyzed with the ImageJ software. The percentage of osteo-differentiated MSC at the different conditions was calculated (f) [ns- P value non-significant] n=2 donors.

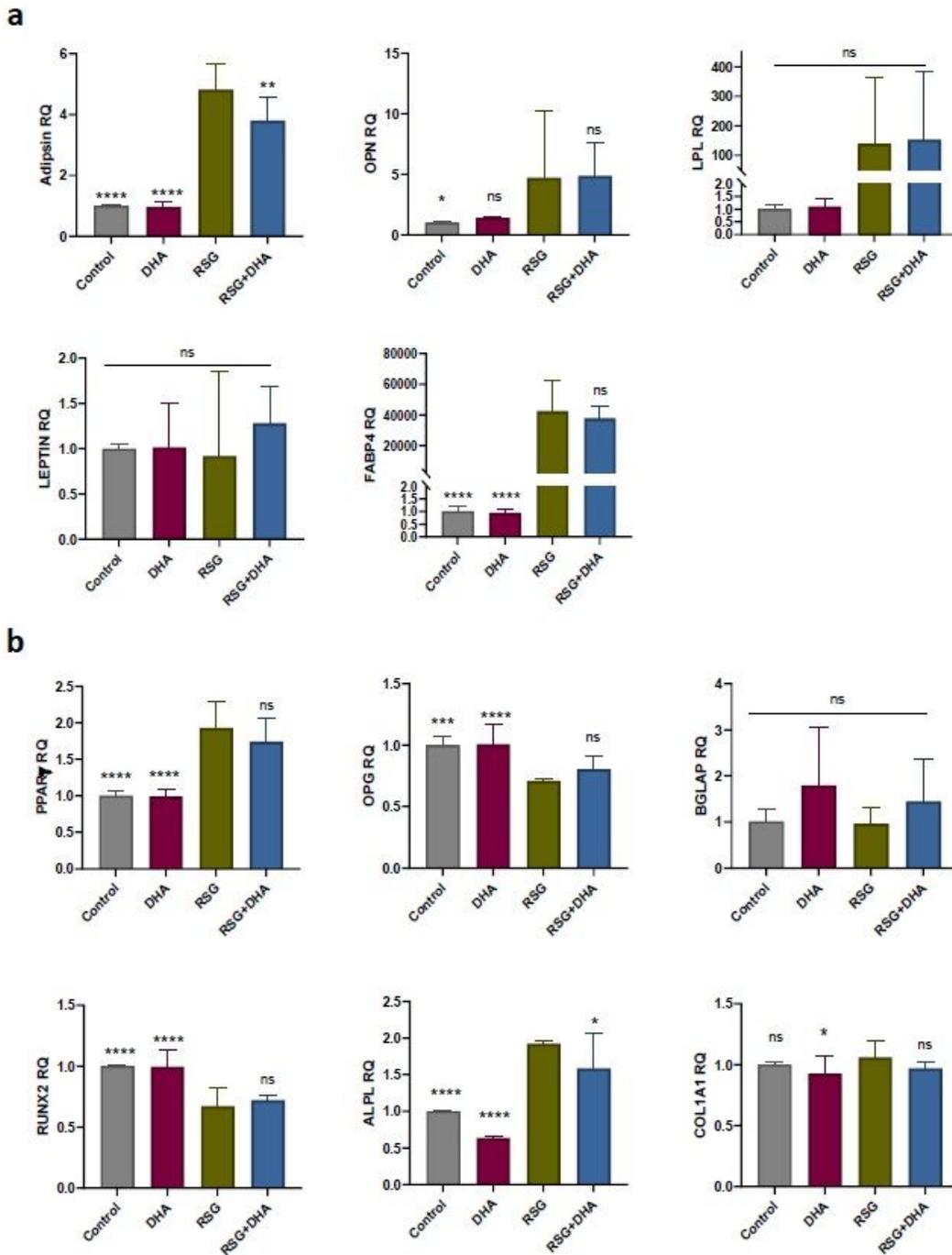


Figure 7

Effect of RSG with or without FO on gene-expression of osteo-differentiated Mesenchymal Stromal Cells (MSCs). Gene expression of adipogenic (a) and osteogenic (b) markers in MSC differentiated in osteogenic medium with the presence or absence of RSG, DHA, and combination of RSG and DHA. Relative quantification of gene expression for Adipsin, FABP4, LPL, PPAR γ , Leptin, OPN, OPG, BGLAP, RUNX2, ALPL and COL1A1. Each bar represents the RQ mean \pm 95% CI from two donors in triplicates. Value with different superscripts is significantly different at $p < 0.05$ by one way ANOVA test followed by Dunnett's multiple comparisons tests with RSG group. ns- P value non-significant.

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