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PRINCIPAL INVESTIGATOR: Dr. Gabriel Fernandes

CONTRACTING ORGANIZATION: University of Texas Health Science Center
San Antonio, TX 78229

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14. ABSTRACT

An estimated 192,370 women in USA will be diagnosed with breast cancer and 40,170 will die in 2009. Further, there were approximately 2,533,193 women in USA living with breast cancer in 2006. Among them, many will develop bone metastasis and will have poor quality of life each year. Metastatic bone disease is a major cause of morbidity in breast cancer patients. It is suspected that the microenvironment of the bone may be influenced by dietary factors and adiposity in the bone marrow may influence bone metastasis and osteolysis.

We have observed that corn oil enriched diet increases obesity and also adipocytes in bone marrow while omega-3 fatty acids show reduced adipocytes and inflammation. We therefore carried out studies using omega-3 fatty acids both *in vitro* and *in vivo*. Our recent studies on proliferation of MDA-231BO cells demonstrated a dose dependent inhibition of tumor cells *in vitro* by EPA and DHA, the latter being more pronounced in its activity. Similarly, matrigel invasion of MDA-231BO breast cancer cells showed significant reduction of tumor cell invasion by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Based on these encouraging observations, we carried out a new study using concentrated EPA (EPA 55% and DHA 5%) and DHA (DHA 60% and EPA 5%) obtained from Ocean Nutrition, Canada. We fed 4 week old nude mice with regular control chow diet, 10% EPA and 10% DHA for 1 month (n=10). Mice in each group were injected with 1×10^5 MDA-231BO breast cancer cells intracardially.

After four weeks, all mice were scanned by X-ray to measure bone osteolysis followed by histomorphology of both leg joints to evaluate tumor proliferation. It was noted that mice fed DHA show very minimal proliferation and osteolysis. Also, osteolysis in the tibial metaphysis was significantly lower in DHA fed mice. The tumor cell proliferation in tibial metaphysis was measured and tumor cell proliferation was markedly lower in DHA fed mice. Further, CD44 expression in bone section was also found lower in DHA fed mice. Thus we are very encouraged to see these results for the first time using intracardial injection of tumor cells, which selectively metastasizes in bone, and the inhibition of its metastasis and osteolysis by DHA. This finding may lead to set up clinical studies in breast cancer patients to prevent onset of metastasis and bone pain in later years.

15. SUBJECT TERMS

Fish oil, Lovaza, Bone Pain, Osteolysis, DHA, fatty acids, nude mice

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INTRODUCTION

It is estimated that 192,370 women in USA will be diagnosed with, and 40,170 women will die of, breast cancer in 2009. Many studies have shown fish oil fatty acids, particularly DHA, to have a protective effect against many cancers, including breast, colon, prostate and ovary [1]. However, the mechanisms underlying this protective effect is not well understood, but may involve induction of apoptosis and inhibition of cell growth, including metastasis. Earlier studies have shown that feeding mice with diets rich in fish oil or its component fatty acids, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), protects against inflammation, osteoclastogenesis, bone loss, autoimmune diseases and most importantly, breast cancer cell proliferation [2-4].

We carried out studies using fish oil (omega-3 fatty acids) both *in vitro* and *in vivo* in order to:

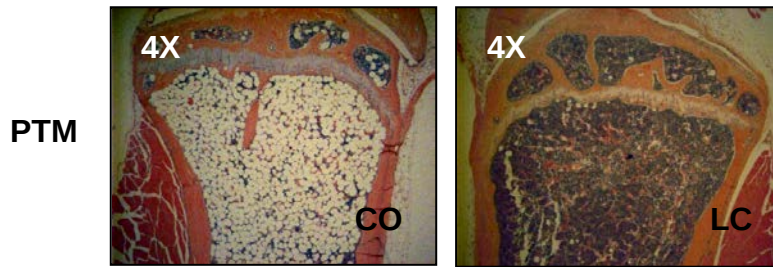
- A. To study the effects of a high fat diet on bone marrow adiposity in C57BL/6 mice by H/E staining of long bones of mice on corn oil (high fat) diet versus standard lab chow (normal fat) mice *in vivo*.
- B. To study the effects of regular fish oil (n-3 fatty acids) versus corn oil (n-6 fatty acids) fed C57BL/6 mice on the propensity of bone marrow adipocytes *in vivo*.
- C. To study the effects of high fat diet on cancer induced pain sensitivity in C57BL/6 mice intra-tibially injected with MDA-231 BO human breast cancer cells on corn oil (high fat) versus standard lab chow (normal fat) mice *in vivo*.
- D. To study the dose dependent effects of fish oil (DHA or EPA enriched) on the proliferation of MDA-231 BO human breast cancer cells *in vitro*.
- E. To study the dose dependent effects of fish oil (DHA or EPA enriched) on invasion through Matrigel of MDA-231 BO human breast cancer cells *in vitro*.
- F. To study the effects of fish oil (DHA or EPA enriched) diets on the propensity of bone metastasis of MDA-231 BO human breast cancer cells intra-cardiac injected to female nude mice *in vivo* and evaluating long bone tumor burden by radiograph, H/E and CD44 staining.
- G. To study the effects of fish oil (various enriched) diets on the propensity of bone metastasis of MDA-231 BO human breast cancer cells injected intra-cardiac to female nude mice *in vivo* and evaluating long bone tumors by radiograph and H/E.
- H. To study the effects of regular fish oil and Omacor (FDA approved prescription fish oil) on the pain sensitivity (hind limb plantar pain sensitivity, RT-PCR expression of hind limb DRG pain sensing genes, and hind limb DRG c-fos expression) in C57BL/6 mice fed enriched fish oil diets for 6 months .

BODY

- A. To study the effects of a high fat diet on bone marrow adiposity in C57BL/6 mice by H/E staining of long bones of mice on corn oil (high fat) diet versus standard lab chow (normal fat) mice *in vivo*.**

In order to determine whether increased bone marrow adipocytes are a result of a high fat diet, C57BL/6 mice were fed either 10% corn oil (high fat supplemented diet) versus lab chow (normal fat diet) for 6 months. Mice were sacrificed, and tibiae bones were formalin fixed and stained for H/E. Bone marrow adipocytes were measured by histomorphometry in the proximal tibial metaphysis (PTM). As seen in **Figure 1**, a high fat diet (corn oil) creates increased adiposity in the bone marrow.

FIGURE 1



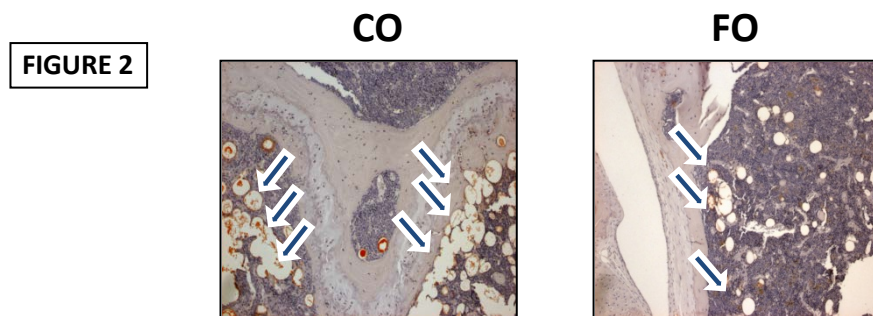
Groups	CO (Obese)		LC (Lean)	
Region of interest /Parameter	Area (μm^2)	OD	Area (μm^2)	OD
PTM	221871.2 $\pm$ 88.3 ^a	462.0 $\pm$ 23.4 ^a	2070.1 $\pm$ 38.4 ^b	131.7 $\pm$ 14.5 ^b

Total adipocytes area in proximal end of tibial expressed as area in (μm^2) and optical density (OD) in CO fed obese and LC fed lean female C56Bl/6J aging mice. Results are expressed as mean $\pm$ SEM, Means in row with superscripts without a common letter significantly different ($P < 0.05$ CO Vs LC) students t-test (unpaired) (n=6-8).

B. To study the effects of regular fish oil (n-3 fatty acids) versus corn oil (n-6 fatty acids) fed C57BL/6 mice on the propensity of bone marrow adipocytes *in vivo* by Oil Red O staining.

C57BL6 mice were either fed a n-3 fatty acid diet (10% regular fish oil 18/12) or a n-6 fatty acid diet (10% corn oil) for 6 months. Animals were sacrificed and tibial bones were collected to evaluate adipocytes. CO fed mice exhibited a marked increase of long bone adipocytes as compared to regular fish oil fed mice as seen in **Figure 2**.

Oil Red O Staining in Long Bones of FO Fed Mice



Oil Red O staining of long bones from mice fed fish oil diets. Photomicrographs are representative photos from each diet group.

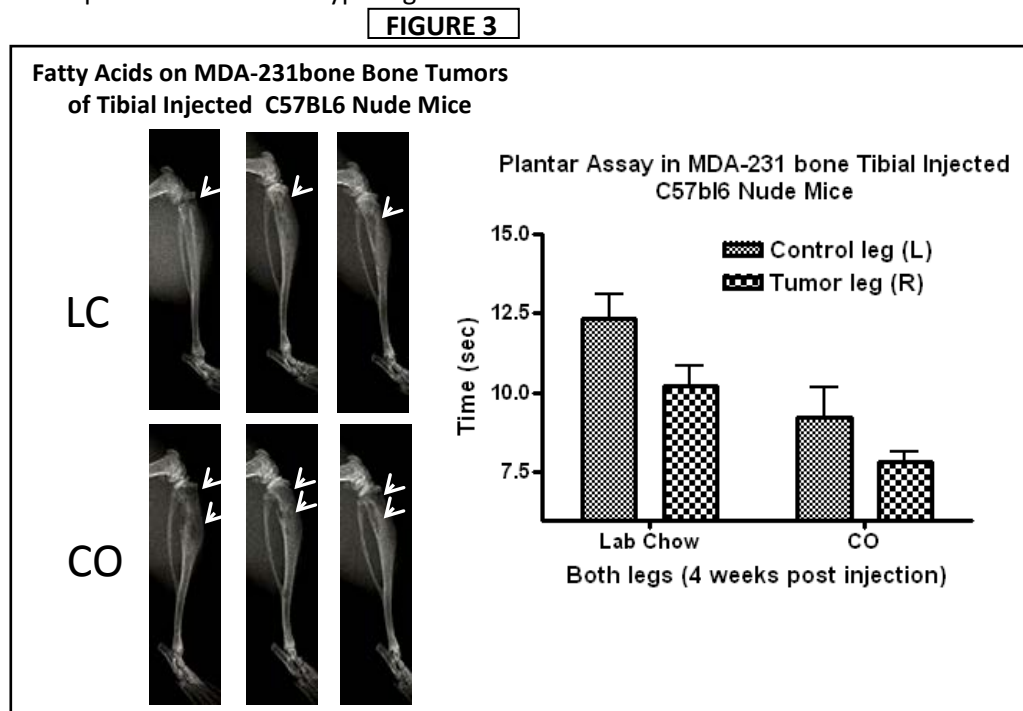
C. To study the effects of high fat diet on cancer induced pain sensitivity in C57BL/6 mice intra-tibially injected with MDA-231 BO human breast cancer cells on corn oil (high fat) versus standard lab chow (normal fat) mice *in vivo*.

Consequently, female C57BL/6 mice were tibially injected with MDA-231 BO cells (1×10^6 / 10ul) and mice were sacrificed after approximately 4 weeks. Prior to sacrifice, mice were subjected to heat sensitivity assay (Plantar Assay) to evaluate pain sensitivity in the tumor leg versus control leg (non-injected leg). As seen below, mice fed a high fat diet, corn oil (CO), experience more tumor bone pain as compared to lab chow (LC) fed mice. As seen in **Figure 3**, the radiographs, increased osteolytic bone metastases is seen in the CO fed mice versus the LC fed mice.

The method for Paw withdrawal latency (Plantar test) is described. Paw withdrawal latency to thermal stimuli of radiant heat was measured using a device (Plantar test, 7370; Ugo Basile, Comerio, Italy). Briefly, animals were placed in an acrylic box with a glass pane floor and the plantar surface of the hindpaw was exposed to a beam of infrared radiant heat. The paw withdrawal latencies were measured three times per session, separated by a minimum interval of 30 min. Paw withdrawals due to locomotion or weight shifting were not counted, and the trials were repeated. Data are expressed as paw withdrawal latency in seconds.

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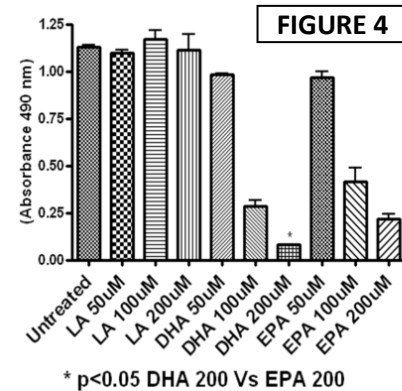
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- II. Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32:77–88.



Left panel: Tibial injection of 1×10^6 MDA-231 bone cells to C57black 6 nude mice after 4 weeks on fish oil diets. Bone tumors are seen after 4 weeks post tumor injection (after 8 weeks on fish oil diets). Right panel: Plantar assay of the right leg (tumor) and left leg (control) of these same mice. Note the increased Pain sensitivity in the Corn Oil group as compared to the Lab Chow group.

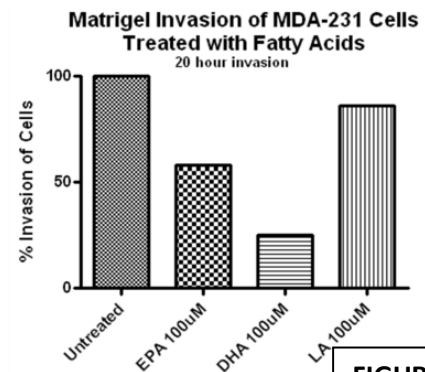
D. To study the dose dependent effects of fish oil (DHA or EPA enriched) on the proliferation of MDA-231 BO human breast cancer cells *in vitro*.

Our recent studies on MTS proliferation of MDA-231BO cells demonstrated a dose dependent inhibition of tumor cells *in vitro* by EPA and DHA (treated for 72 hours), the latter being more pronounced in its activity. MTS Assay procedures were followed according to the manufacturer's instruction (Promega). Cells (2×10^4) were plated on 96 well plates and incubated for 72 hours in the presence of various concentrations of fatty acids. LA was used as a control fatty acid in respect to DHA and EPA as seen in **Figure 4**.



E. To study the dose dependent effects of fish oil (DHA or EPA enriched) on invasion through Matrigel of MDA-231 BO human breast cancer cells *in vitro*

Similarly, Matrigel invasion of MDA-231BO breast cancer cells showed significant reduction of tumor cell invasion by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Cells (5×10^4) were plated on 24 well plate Matrigel inserts and allowed to transmigrate for 20 hours. Inserts were then fixed and stained with crystal violet and transmigrated cells counted. DHA significantly reduced tumor cell migration as compared to EPA as seen in **Figure 5**.



Based on these encouraging observations, we carried out a new study using concentrated EPA (EPA 55% and DHA 5%) and DHA (DHA 60% and EPA 5%) obtained from Ocean Nutrition, Canada.

F. To study the effects of fish oil (DHA or EPA enriched) diets on the propensity of bone metastasis of MDA-231 BO human breast cancer cells intra-cardiac injected to female nude mice *in vivo* and evaluating long bone tumor burden by radiograph, H/E and CD44 staining.

We next fed 4 week old nude mice with regular control chow diet, 9% EPA/1% CO and 9% DHA1% CO (**Table 1**) for 1 month (n=10 mice). All the mice were housed 5/cage at $22^\circ\text{C} \pm 0.5^\circ\text{C}$ on 12-hour dark/light cycle. Mice in each group were injected with 1×10^5 MDA-231BO breast cancer cells intracardially.

After four weeks, all mice were scanned by X-ray to measure bone osteolysis followed by histomorphology of both leg joints to evaluate tumor proliferation. It was noted that mice fed DHA show very minimal proliferation and osteolysis. Also, osteolysis in the tibial metaphysis was significantly lower in DHA fed mice as seen in **Figure 6A**. The tumor cell proliferation in tibial metaphysis

TABLE 1

Composition of different oils/FAs in AIN-93 diet.		
Ingredients	9% DHA	9% EPA
Casein	140.0	140.0
L-lysine	1.8	1.8
Corn starch	175.0	175.0
Dextrinized corn starch	424.7	424.7
Sucrose	90	90
Corn Oil	10.0	10.0
DHA (5/60)	90.0	0.0
EPA (55/5)	0.00	90.0
Cellulose	50.0	50.0
AIN-93 mineral mix	35.0	35.0
Choline bitartrate	2.5	2.5
TBHQ	1.0	1.0
Ain-93 vitamin mix	10.0	10.0
No. of mice/group	10	10

Control group (n=10) was maintained on standard Lab chow (LC). DHA and EPA were obtained from Ocean Nutrition, Canada.

Inhibition of Bone Metastasis by DHA and EPA

FIGURE 6A

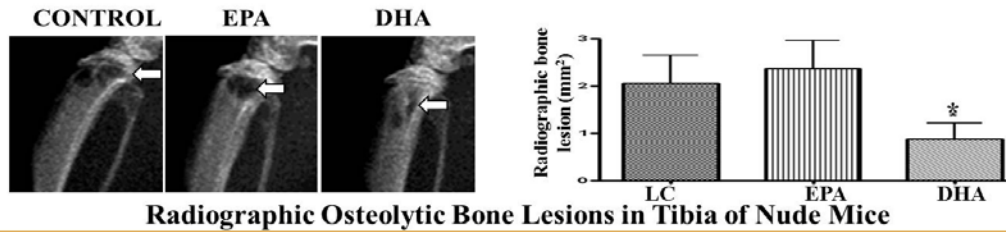


FIGURE 6B

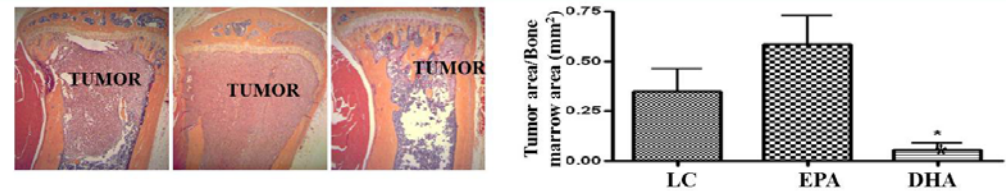
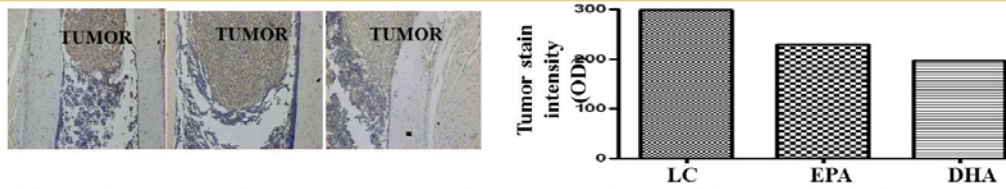


FIGURE 6C



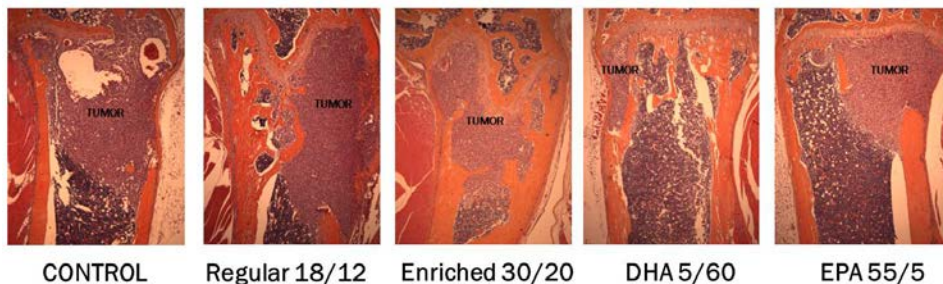
was measured and tumor cell proliferation was markedly lower in DHA fed mice as seen in **Figure 6B**. Furthermore, CD44 expression in bone sections were also found lower in DHA fed mice as seen in **Figure 6C**. Thus we are very encouraged to see these results for the first time using intracardial injection of tumor cells, which selectively metastasizes in bone, and the inhibition of its metastasis and osteolysis by DHA.

- G. To study the effects of fish oil (various enriched) diets on the propensity of bone metastasis of MDA-231 BO human breast cancer cells injected intra-cardiac to female nude mice *in vivo* and evaluating long bone tumors by radiograph and H/E.

Inhibition of Bone Metastasis by Various Fish Oils

H&E Staining of Bone Lesions in Tibia of Nude Mice

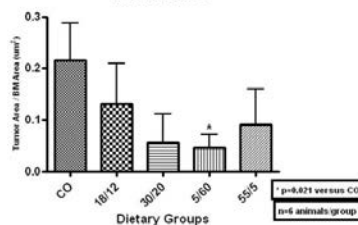
FIGURE 7A



Effect of Fish Oils on MDA-231BO Long Bone Tumors in Nude Mice

✓Nude mice intracardiac injected with 1×10^5 human breast cancer cells.

✓Radiographs at 4 weeks post injection.



Inhibition of Bone Metastasis by Various Fish Oils

Radiographic Osteolytic Bone Lesions in Tibia of Nude Mice

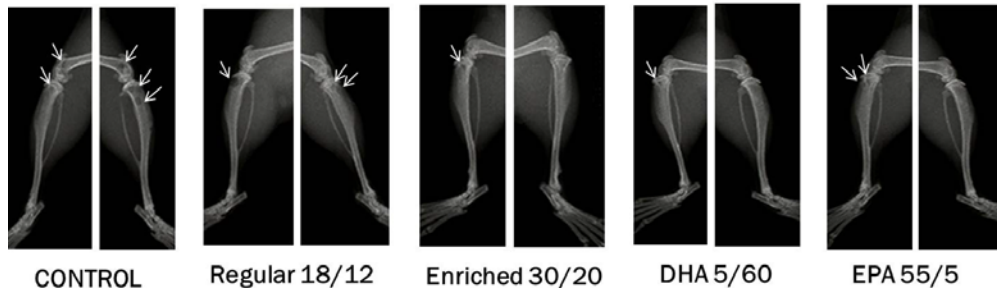
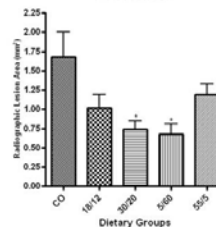


FIGURE 7B

✓Nude mice intracardiac injected with 1×10^5 human breast cancer cells.

✓Radiographs at 4 weeks post injection.

Effect of Fish Oils on MDA-231BO Osteolytic Bone Lesions in Nude Mice



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DHA 5/60 fish oil significantly reduces long bone tumor area as compared to EPA 55/5. Regular fish oil 18/12 also is found to reduce long bone tumor volume as compared to control fed mice (corn oil), though not as significantly as DHA 5/60, as seen in **Figure 7A** and **Figure 7B**.

H. To study the effects of regular fish oil and Omacor (FDA approved prescription fish oil) on the pain sensitivity (hind limb plantar pain sensitivity, RT-PCR expression of hind limb DRG pain sensing genes, and hind limb DRG c-fos expression) in C57BL/6 mice fed enriched fish oil diets for 6 months.

Isolation of mouse L3-L5 DRG's were carried out using the following method: Fresh mouse L3-L5 dorsal root ganglions (DRGs) were isolated. In brief, mice were laid in the prone position and the spinal cord and the segmental distribution of the DRG was exposed by making an incision across the spine. Parallel cuts were then made through the vertebrae adjacent to the spinal cord, and the overlying muscle and bone were removed. The last thoracic ganglion (T13) and the ganglion with the greatest contribution to the sciatic nerve (L4) were identified for orientation with respect to the whole mice. The dorsal roots were very short at the cervical and upper thoracic levels, and the ganglia lie closely apposed to the spinal cord. L3-L5 DRG's were removed, and placed in cold saline solution. DRG's were then processed either for RT-PCR, hematoxylin/eosin or c-fos staining. Reference: Malin S, et al. (2007) Production of dissociated sensory neuron cultures and consideration for their use in studying neuronal function and plasticity. Nature Protocols 2: 152-60.

Immunohistochemistry of c-Fos and Hematoxylin/Eosin Staining of mouse DRGs was carried out in mouse spinal cord segments (DRGs) at lumbar level L3–L5 that were removed, fixed in 10% formalin for 24 h and processed and embedded in paraffin. Serial sections (5µm thick) were made using a sliding microtome. Standard hematoxylin and eosin staining was performed. c-Fos immunostaining was

performed using rabbit anti-human c-fos antibody (sc-52, Santa Cruz Technologies) at 1:50 dilution for 1 hour RT. Development was carried out using the appropriate Vector Stain kit (Vector Labs, Burlingame, CA), and counterstained with hematoxylin. Photomicrographs were carried out using a light microscope. Fish oil fed mice (Lovaza) exhibited lower c-fos staining in isolated DRG from mice as seen in **Figure 8A**. In addition, pain

Fish Oil in Possible Pain Sensitivity in Mice

RT-PCR Analysis of Acid-Sensing Pain Genes in L3-L5 Isolated DRGs of Concentrated Fish Oil Diet Fed C57bl6 Female Mice

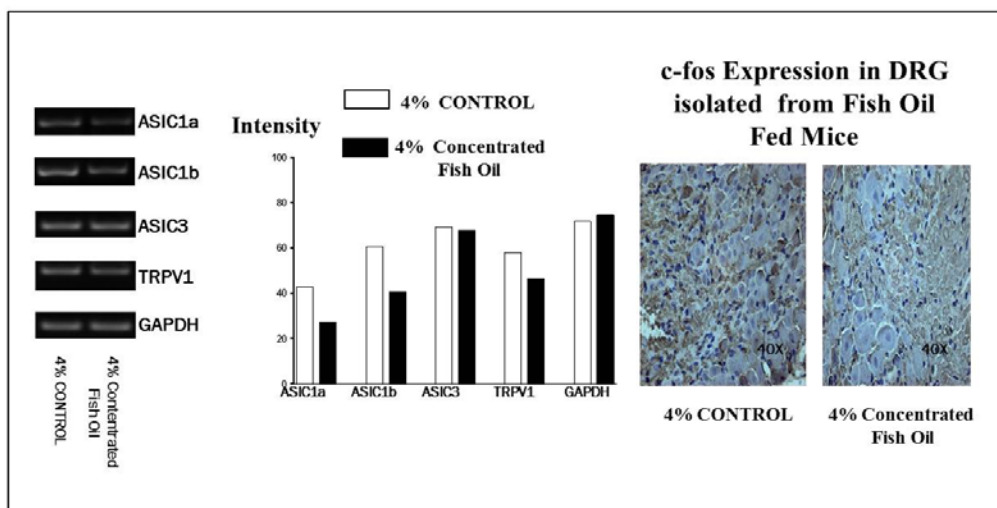


FIGURE 8A

Left panel: RT-PCR of pain genes in isolated DRGs of concentrated fish oil (Lovaza) fed C57bl6 mice. Relative expression intensity is shown in the graph.
Right panel: c-fos immunostaining of the same DRG's isolated from these mice. Note the increased staining in the placebo group compared to the concentrated fish oil group.



Reverse transcriptase-polymerase chain reaction (RT-PCR) with mouse DRG was carried out using the following method: Fresh mouse dorsal root ganglions (DRGs) (L3–L5) were dissected following the protocol as described (1). In brief, total RNA was isolated using RNeasy Mini-kit (Qiagen, Valencia, CA), and single-strand cDNA was synthesized from 0.4ug RNA using Superscript III first-strand kit (Invitrogen, Carlsbad, CA). RT-PCR was carried out using HotStarTaq Master Mix kit (Qiagen, Valencia, CA). The primer sets used for PCR were as follows:

rat/mouse ASIC1a, CACAGATGGCTGATGAAAAGCAG/CATGGTAACAGCATTGCAGGTGC (30 cycles);
 rat/mouse ASIC1b, ATGCCGT GCGGTTGTCCC/CATGGTAACAGCATTGCAGGTGC (30 cycles);
 mouse ASIC3, TGAGAGCCACCAGCTTACCT/ACATGTCCTCAAGGGAGTGG (30 cycles);
 mouse TRPV1, GTGACCCTCTTGGTGGAGAA/CTTCAGTGTGGGGTGGAGTT (30 cycles),
 mouse GAPDH, GGTGAAGGTCGGTGTGAACG/CTCGCTCCTGGAAGATGGTG (30 cycles).

PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light. The size of the fragments was confirmed by reference to a 100-bp DNA ladder. Quantification of amplified mRNA was done by densitometry assisted by the image analysis software MetaMorph Image (xxxxx). Sizes are as follows: ASIC1a 506bp, ASIC1b 563bp, ASIC3 245bp, TRPV1

324bp, and GAPDH 233bp as seen in **Figure 8A**. Reference: Malin S, et al. (2007) Production of dissociated sensory neuron cultures and consideration for their use in studying neuronal function and plasticity. Nature Protocols 2: 152-60.

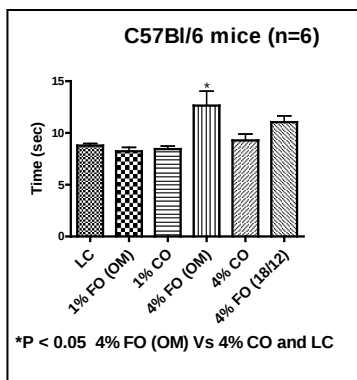


FIGURE 8B

Paw withdrawal latency (Plantar test) performed on C57BL/6 mice showed decrease pain sensitivity in fed 4% Lovaza (OM) as compared to 4% regular fish oil (18/12) and lab chow fed mice for 6 months as seen in **Figure 8B**.

KEY RESEARCH ACCOMPLISHMENTS

- ✳ High fat diet (corn oil) significantly increased bone marrow adiposity in mice *in vivo*.
- ✳ High fat diet (corn oil) significantly increased breast cancer induced bone pain sensitivity in mice *in vivo*.
- ✳ DHA is more potent than EPA in reducing proliferation and Matrigel invasion of MDA-231 BO human breast cancer cells *in vitro*.
- ✳ DHA is more effective than EPA and regular fish oil in reducing MDA-231 BO breast cancer bone metastases and osteolysis in female nude mice *in vivo*, including decreased CD44 expression.
- ✳ FDA prescribed fish oil, Lovaza (OM) reduces hindlimb pain sensitivity and reduces pain sensing gene expression in DRG's of C57BL/6 mice *in vivo*.

REPORTABLE OUTCOMES

The summary of this work was presented during the “Era of Hope” Breast Cancer Research Conference, held in Orlando, Florida at the Orlando World Centre Marriot from August 2-5, 2011. (Abstract # P39-4 entitled “Omega-3 Docosahexaenoic Acid Prevents Breast Cancer-Induced Bone Metastasis and Osteolysis in Nude Mice)

CONCLUSION

DHA omega-3 fish oil is more potent in reducing the growth, invasion and osteolysis of human MDA-231 BO breast cancer cells, both *in vivo* and *in vitro* than EPA and regular fish oil. Preliminary studies performed in evaluating the effect of prescription concentrated fish oil (Lovaza) in reducing pain sensitivity and pain sensing gene expression is promising. Additional studies need to be carried out in order to evaluate the potential effect Lovaza may have in reducing breast cancer-induced bone pain.

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