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STUDY TITLE: Fish Oil Mediated Cardiovascular Complications in ApoE^{KO} Mice
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SUMMARY OF STUDY

BACKGROUND: Atherosclerosis is the principle cause of cardiovascular disease. Due to their spontaneous development of atherosclerosis, apolipoprotein E knockout mice (ApoE^{KO}) are one of the best studied animal models of atherosclerosis. Although previous reports have evaluated the effects of omega-3 fatty acids in the prevention of cardiovascular disease, little is known on their effects in the setting of ApoE deficiency.

OBJECTIVE: To assess the cardiovascular effects of omega-3 fatty acid supplementation (fish oil, flaxseed oil, and designer oil) in ApoE^{KO} mice maintained on a high fat diet.

METHODOLOGY: A total of 40 six-week old ApoE^{KO} mice were randomized into 4 treatment groups. All animals were fed a Western-type diet reconstituted with either safflower oil (control), fish oil, flaxseed oil or designer oil. *In vivo* cardiac function was assessed weekly using echocardiography. Blood pressure and plasma lipid levels were serially measured. Cardiac remodeling was examined using histological analysis and biochemical assessment of a cardiac biomarker: brain natriuretic peptide (BNP).

RESULTS: Echocardiography demonstrated increased ventricular wall thickness in the fish oil treated mice, as compared to the control group ($p<0.05$). Blood pressure increased over time in the fish oil treated mice ($p<0.05$). Relative to control, the fish oil group also showed increased plasma triglycerides ($p<0.01$), and decreased plasma total cholesterol ($p<0.05$). Furthermore, ventricular BNP expression was elevated in the fish oil treated mice ($p<0.05$). Flaxseed oil and designer oil produced similar cardiovascular effects as the control diet.

CONCLUSION: A high fat diet supplemented with fish oil leads to adverse cardiovascular effects in ApoE deficient mice.

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INTRODUCTION

Background Knowledge

Atherosclerosis is the principle cause of ischemic heart disease and stroke, and contributes to about 50% of all deaths in the developed countries.(1) Risk factors for developing atherosclerosis include genetic predisposition, age, male gender, hypertension, diet with high levels of saturated fat, and a sedentary lifestyle.(2) Atherosclerotic lesions may chronically occlude blood vessels or acutely rupture, leading to a range of clinical disorders including cardiovascular disease, cerebrovascular disease and peripheral vascular disease.(1, 2) In order to study the pathogenesis and management of atherosclerosis, numerous animal models have been created to emulate the disease features observed in humans.

ApoE Knockout Model of Atherosclerosis

Due to their spontaneous development of atherosclerosis, apolipoprotein E knockout mice ($ApoE^{KO}$) are one of the best studied animal models of atherosclerosis.(1, 3-5) ApoE is a signaling molecule that resides on lipoproteins such as chylomicron and very low density lipoprotein (VLDL) particles.(3) These lipoproteins carry cholesterol and triglyceride throughout the body.(3) ApoE serves as a ligand for binding to low density lipoprotein (LDL) receptor and LDL receptor related proteins, a process that facilitates the uptake of lipid particles from the circulation into cells.(4, 5) $ApoE^{KO}$ mice express features including hypertriglyceridemia, hypercholesterolemia and elevated inflammatory cytokines, all of which contributes to the development of severe atherosclerosis.(6-8) The atherosclerotic process can be accelerated by consumption of a high fat, high cholesterol diet.(5) Conversely, the atherosclerotic process can be mitigated or even reversed using dietary “healthy fats”, also known as nutraceuticals.(8)

Essential Fatty Acids as Nutraceuticals

In North America, the most prevalent diet is a high fat, high cholesterol diet, commonly referred to as the Western diet. Humans require the intake of omega-3 (n-3) and omega-6 (n-6) essential fatty acids for maintenance of health.(8) However, the “Western diet” intake of n-3 fatty acids are often inadequate.(9)

Omega-3 fatty acids consist of 3 main types: alpha-linoleic acid, (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).(10) They are present in varying fractions in plants and animal lipids. Flaxseed primarily contains the ALA variety of n-3 fatty acid, whereas fish oil contains DHA and EPA.(10) The variations in fatty acid constitution have been demonstrated to produce different cardiovascular effects, though the mechanism is unknown.(11) Oils containing high levels of n-3 fatty acids, including flaxseed and fish oil, have been shown to have anti-hypertensive, anti-inflammatory and anti-atherosclerotic properties.(12-15) Currently, it is believed that n-3 fatty acids act

through multiple mechanisms, including: (1) modifying plasma lipid profile; (2) modulating inflammatory response; (3) lowering blood pressure; (4) and improving endothelial function.(16-18) Despite our knowledge of their physiological actions, the molecular mechanism of n-3 fatty acids are still subject to debate.

Unlike n-3 fatty acids, n-6 fatty acids are rich in the Western diet. Safflower oil, which contains almost exclusively n-6 fatty acids, is often incorporated in laboratory protocols to emulate the Western diet, and thus serves as the dietary control. Excess intake of n-6 fatty acids negatively impacts vascular health, and has been implicated in the increasing prevalence of cardiovascular disease in developed nations.(9) The current paradigm suggests that a balanced intake of n-6 and n-3 fatty acids is important for cardiovascular health, though an optimal ratio of n-6:n-3 fatty acids has not been established.

We have recently developed a designer oil formulation containing a low ratio of n-6:n-3 fatty acids, while maintaining a balance of ALA, EPA and DHA.(19) Low n-6:n-3 diets supplemented with n-3 fatty acids have previously been shown to decrease plasma triglycerides and plasma cholesterol, and reduce atherosclerosis in mice maintained on an atherogenic diet.(19, 20) Using the $ApoE^{KO}$ model of atherosclerosis, we intend to further investigate the cardiovascular effects of dietary supplementation with various n-3 fatty acids.

OBJECTIVES

To determine the cardiovascular effects of omega-3 fatty acid supplementation (fish oil, flaxseed oil, and designer oil) in $ApoE^{KO}$ mice fed a high fat diet.

METHODOLOGY

Animals and Diets. All animal procedures were conducted in accordance with guidelines published by the Canadian Council on Animal care. The study protocol was approved by the Institutional Animal Care Committee on the use of animals in research at the University of Manitoba.

A total of 40 six-week old male $ApoE^{KO}$ mice (B6.129P2-$ApoE^{+m1Unc/J}$, Jackson Laboratories, Bar Harbor, ME) were randomized into 4 treatment arms: safflower oil (control group) (Loblaw, Canada) (n=10); fish oil (ProNova BioCare, Norway) (n=10); flaxseed oil (Dyets Inc. Bethlehem, PA) (n=10); or designer oil (a mixture of safflower, flaxseed, fish oil, and beef tallow) (n=10). All $ApoE^{KO}$ mice were fed a Western-type diet (42% of total calories from fat; 42.7% from carbohydrates; 15% from protein; and 0.1% cholesterol), using standard murine chow (Mouse Diet 9F 5020, PMI Nutritional International, Richmond, IN) reconstituted with the control and treatment oils (20% wt/wt) (Table 1). All mice were housed under standard conditions (12:12-h light-dark cycle, temperature controlled room), with ad libitum access to chow and water.

Murine Echocardiography. *In vivo* assessment of cardiac function was performed using 2-dimensional transthoracic echocardiography. All mice were imaged at baseline, and weekly thereafter for 16 weeks. Murine echocardiography was performed using a 13-Mhz probe (Vivid 7, GE Medical Systems, Milwaukee, WI) in conscious mice, as previously described.(21) Post-processing analysis was made using Echopac PC (version 110.0.0, GE Medical, Milwaukee, WI).

Murine echocardiography was performed in the parasternal long and short axis views. Cardiac dimensions, including posterior wall thickness (PWT), interventricular septal thickness (IVS), Left ventricular end diastolic diameter ($LVID_d$), fractional shortening (FS), and heart rate (HR), were obtained using M mode (Figure 2a). Left ventricular (LV) volume and ejection fraction (LVEF) were calculated using the Teichholz's method (Figure 2b).(21)

Hemodynamics and Plasma Lipids. Blood pressure (BP) and heart rate (HR) were measured using the tail-cuff method (CODA system, Kent Scientific, Torrington, CT) on conscious, restrained mice at baseline, week 8 and week 16, as previously described.(22) Plasma total cholesterol (TC) and triglycerides (TG) were quantified at baseline, weeks 8 and 16 using enzymatic kits following manufacturer's instructions (Sekisui Diagnostics, Japan).

Histology and Biochemical Analysis. After 16 weeks of treatment, the mice were euthanized and the aorta, heart, liver and spleen were collected. Ventricular myocardium was fixed, paraffin embedded, and stained with hemotoxylin and eosin (H&E). Appropriate sections were cut for pathology and morphometric examinations. Apical portion of the ventricles were immediately placed in RNA stabilizing solution after excision (RNAlater, Qiagen, Hilden, Germany), and frozen for further PCR analysis. BNP mRNA expression was assessed using real time quantitative reverse transcription polymerase chain reaction (real time qRT-PCR).

Statistics. All values represent mean ± SEM. Two-tailed Student's t-test and repeated measures analysis of variance (ANOVA) were used to determine the significant differences between the control and treatment groups at the level of $p<0.05$. Statistical analysis was conducted using GraphPad Prism 5 (Graphpad Software, La Jolla, CA).

RESULTS

Echocardiography

The cardiac dimensions were similar amongst the four groups at baseline (Figure 3), and the values were consistent with those previously reported.(23) At week 16, $ApoE^{KO}$ mice treated with fish oil demonstrated significantly increased posterior wall thickness (1.19±0.03 vs. 0.92±0.04mm in control group, $p<0.05$) and interventricular septum thickness (1.10±0.03 vs. 0.85±0.03mm in control group, $p<0.05$) (Figure 3). There was

no significant cardiovascular remodeling in $ApoE^{KO}$ mice treated with either flaxseed oil or designer oil. The left ventricular ejection fraction was not significantly changed over time, and no difference was observed between groups.

Hemodynamics

At baseline, the heart rate and blood pressure were comparable amongst the four groups (Figure 4), and the values were similar to those previously reported.(24) The systolic BP, diastolic BP and mean arterial pressure increased over time in the fish oil treatment group ($p<0.05$). No other group showed changes in BP over time. Furthermore, there were no hemodynamic differences between the four groups at any time point. However, HR was significantly decreased in fish oil treated mice at all time points, compared to the control group ($p<0.05$).

Plasma Lipid Profile

At baseline, the plasma total cholesterol levels were similar amongst the four groups, and were consistent with previous reports.(25) Compared to baseline, TC significantly increased over time in $ApoE^{KO}$ mice treated with safflower oil, flaxseed oil and designer oil (Figure 5a). There was no difference between these treatment groups, at any time. In contrast, plasma TC did not increase significantly in the fish oil group over time. Furthermore, relative to the control group, the fish oil fed mice had significantly lower plasma TC at week 8 (641±66 vs. 846±76mg/dL in control group, $p<0.05$) and week 16 (421±32 vs. 713±46mg/dL in control group, $p<0.05$).

Like TC, the plasma triglyceride levels were comparable amongst the four groups at baseline, and were similar to those previously reported.(25) At weeks 8 and 16, plasma TG levels significantly increased in all groups, compared to baseline ($p<0.05$ for all groups) (Figure 5b). Relative to control, fish oil demonstrated a significant increase in plasma TG at week 8 (271±28 vs. 118±9 mg/dl, $p<0.01$). There was no statistically significant difference between the control group, flaxseed oil and designer oil throughout the course of the experiment.

Histology

Preliminary histology report suggests a subtle trend towards left ventricular hypertrophy (LVH) in the fish oil treated group (Figure 6). The current report is based on n=5 per group. Histology investigations for the remaining mice are ongoing.

Biomarker: Brain natriuretic peptide

At the end of the experiment, expression of BNP in the myocardium was similar amongst flaxseed oil, designer oil and safflower oil treated mice (Figure 7). The fish oil

treated group demonstrated 46±17% increase ventricular BNP expression relative to the control ($p<0.05$).

DISCUSSION

This study characterized the effects of n-3 fatty acids on the cardiovascular function of $ApoE^{KO}$ mice. Echocardiography demonstrated thickening of the left ventricular walls in the fish oil treated mice ($p<0.05$), as compared to the safflower (control) group. In terms of hemodynamics, blood pressure increased over time in the fish oil group ($p<0.05$). Compared to control, mice treated with fish oil demonstrated decreased TC at week 8 and 16 ($p<0.05$), and increased TG at week 8 ($p<0.01$). Ventricular BNP expression was also significant higher in the fish oil group, relative to the control ($p<0.05$). $ApoE^{KO}$ mice treated with either flaxseed or designer oil produced similar cardiovascular effects to the control group.

The increase in plasma TG in all treatment groups over time is likely the result of a high fat diet, and the lack of functional ApoE to facilitate clearing of plasma lipids (Figure 6). While plasma TG significantly increased over time in all four groups, only fish oil was statistically different than the control group. The elevated plasma TG at week 8 (270mg/dl) in fish oil treated mice is consistent with previous reports (11, 25, 26), which also observed similar levels of plasma TG after fish oil treatment in $ApoE^{KO}$ mice. In contrast, numerous studies have reported that fish oil treatment in ApoE competent mice decreases plasma TG over time.(11, 19, 20) The paradoxical effect of fish oil in $ApoE^{KO}$ mice suggests that functional ApoE is essential in mediating the TG lowering effect of fish oil. Recently, it has been shown that fish oil disrupts the VLDL interaction with lipoprotein lipase (LPL)(11), which is a physiological mechanism of TG clearance. Although the disruption occurs in both wild type and $ApoE^{KO}$ mice, functional ApoE allows wild type mice to overcome the inhibition. In contrast, the inhibition cannot be overcome in ApoE deficient mice, consequently hypertriglyceridemia results. Thus, the elevated TG in response to fish oil appears to be genotype dependent.

Similar to plasma TG levels, most treatment groups (safflower oil, flaxseed oil and designer oil) demonstrated increased plasma TC over time ($p<0.05$ for all groups at week 8 and 16). However, compared to the control group, fish oil treated mice showed significantly lower TC throughout the experiment, and did not show significant increase in TC over time. This result differs from a previous study by Xu et al.(25), where fish oil had no effect on plasma TC of $ApoE^{KO}$ mice compared to the control diet (normal chow). The disparity in results may be attributable to the discrepancies in protocol, including the length of experiment, the control diets, and the fish oil content in the diet. Interestingly, the result from the current study is similar to previous reports in ApoE competent animal models, where TC levels decrease with fish oil treatment.(11, 20) This suggests that the effect of fish oil on plasma TC may be independent of the ApoE pathway. Currently, it is unclear how the n-3 fatty acid diets lower serum cholesterol, whether by inhibiting absorption of dietary cholesterol, increasing biliary excretion, or

increasing cholesterol catabolism. Du et al.(27) suggested that n-3 fatty acids supplemented diet decreases HMG-CoA reductase activity, thereby lowering cholesterol. This mechanism may also apply to the present study.

Surprisingly, flaxseed oil and designer oil did not produce any changes in plasma lipids compared to safflower oil (n-6 fatty acid). Since the n-3 fatty acids in both flaxseed oil and designer oil are highly ALA rich (99% and 84% ALA in flaxseed oil and designer oil, respectively), they likely share a similar mechanism of action. In contrast, the constitution of n-3 fatty acid in fish oil is very different (83% EPA+DHA, 17% ALA). Since fish oil produced vastly different effects from flaxseed oil and designer oil, the results are highly suggestive that EPA and DHA mediate different mechanisms than ALA. This is in concordance with past studies, where different cardiovascular action of EPA, DHA and ALA have been reported.(10, 15, 28, 29) However, the specific actions of each n-3 fatty acid subtype are currently not well understood.

Past reports have suggested that lower n-6:n-3 fatty acid ratio is important to cardiovascular health by favorably modifying tissue and plasma lipid profile.(20, 30) However, the n-6:n-3 fatty acid ratio appears to play little role in this experiment. For instance, safflower oil, flaxseed oil and designer oil had different n-6:n-3 ratios ranging from 0.75 to 20, yet they all had similar cardiovascular effects. We speculate that there may be several reasons why modifying n-6:n-3 fatty acid ratio was inefficacious. However, the most likely reason is that the type of n-3 fatty acid may have much more influence on the cardiovascular system than the n-6:n-3 fatty acid ratio. For instance, in this study, flaxseed and fish oil had similar n-6:n-3 fatty acid ratios (0.75 vs. 0.45, respectively), yet their effects were profoundly different. Thus, the different n-3 contents of safflower oil, flaxseed oil and designer oil may have overshadowed the effect of modifying n-6:n-3 ratio. Indeed, past report have demonstrated that the effect of lowering n-6:n-3 ratio is often subtle.(20)

To further evaluate the cardiac of n-3 fatty acid supplementation, we measured BNP expression in ventricular myocytes. Elevated BNP expression is strongly associated with cardiac dysfunction and hypertrophy.(31, 32) In the present study, the increased ventricular expression of BNP in fish oil treated mice, along with increased LV wall thickness on echocardiography, are both indicative of LVH. The presence of elevated BP could be a causative mechanism of the cardiac remodeling. However, the possibility that EPA and DHA may also have direct effect on cardiomyocyte regulation cannot be excluded. Overall, the hemodynamic, echocardiographic and biochemical evidence all suggest cardiac hypertrophy and remodeling. To our knowledge, this is the first report showing adverse hemodynamic impact and cardiac remodeling due to fish oil treatment.

The results of this study challenge the current perception of the cardioprotective effects of fish oil. Previous reports have demonstrated that fish oil is beneficial to cardiovascular health, both in animal models and clinical studies.(17, 20, 25, 33) Many reports using ApoE competent animal models have shown fish oil to decrease blood

pressure and favorably modulate plasma lipid profile.(19, 20, 28, 34, 35) The opposing effects of fish oil treatment between ApoE competent and ApoE deficient states suggest that functional ApoE may be essential for mediating the beneficial effects of n-3 fatty acids.

STUDY LIMITATIONS

There are a number of limitations in the current study. First, compared to invasive BP monitoring, the use of non-invasive BP monitoring is subject to larger margin of error and less sensitive to small changes in BP.(36) Non-invasive BP measurement was selected because the study required serial measurements. Second, it was difficult to ensure equal doses of the treatment oil were received for each mouse, as the supplement was mixed with the chow. Thus, the intake of oil varied with the chow intake. Third, the sample size limits the statistical power of the study. A larger sample size would allow for better differentiation of statistical differences between the treated and control groups. Finally, the short duration of the experiment may limit the detectability of chronic cardiac changes. As cardiovascular disease develops over time, longer treatment terms may allow for the differences between treatment groups to become more evident.

CONCLUSION

A high fat diet supplemented with fish oil leads to adverse cardiovascular effects in ApoE deficient mice.

CLINICAL SIGNIFICANCE

There is significant ApoE gene polymorphism in the human population. There are 3 ApoE alleles: ApoE2, ApoE3 and ApoE4. Together, these 3 alleles constitute 6 different genotypes, ApoE2/2, ApoE2/3, ApoE2/4, ApoE3/3, ApoE3/4, and ApoE4/4. To our knowledge, no large clinical trials have been performed to study the interaction between ApoE genotype and the response to n-3 supplementation. Though small prospective trials have attempted to address this question (37), our understanding of the interaction between n-3 fatty acid and ApoE genotype is far from complete.

The interaction between nutraceuticals and genetic variations has important clinical implications. For instance, Type 3 hyperlipoproteinemia, a clinical condition commonly associated with ApoE2/2 genotype, is akin to the $ApoE^{KO}$ mouse model used in the present study. In both cases, functional ApoE is absent, which leads to hypertriglyceridemia and atherosclerosis.(38) Since fish oil may have adverse cardiovascular effects in the ApoE deficient genotype, fish oil supplementation in this human population may also have detrimental cardiovascular impact.

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APPENDIX

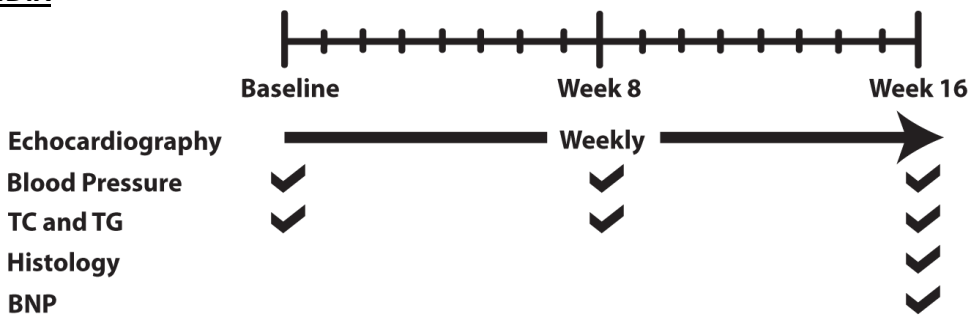

Figure 1. Experimental protocol for all treatment groups is shown. Cardiovascular parameters were tested using five different techniques: (1) echocardiography; (2) tailcuff measurement for blood pressure; (3) colorimetric assays for plasma total cholesterol (TC) and plasma triglycerides (TG); (4) cardiac histology and (5) quantitative real time RT-PCR for ventricular brain natriuretic peptide (BNP) expression.

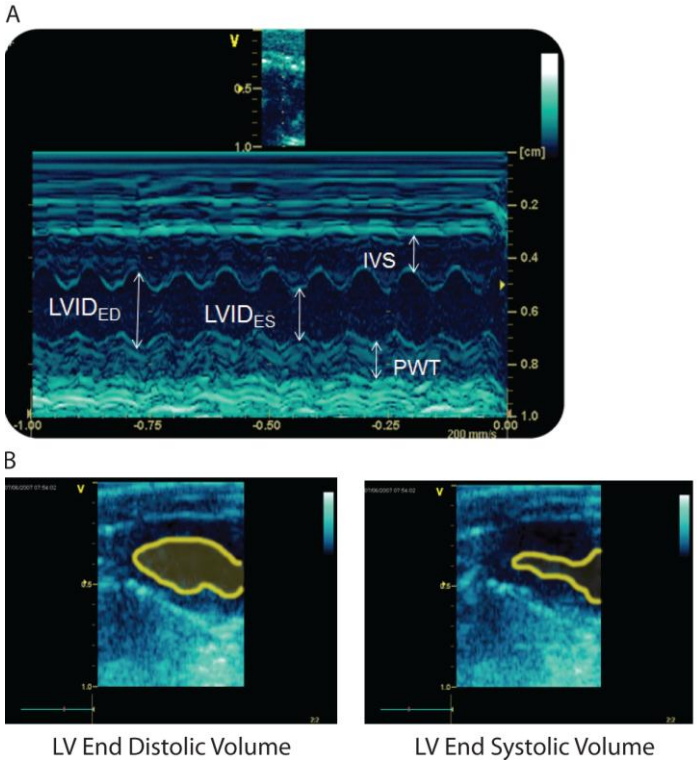

Figure 2.

A. M-mode echocardiography in the parasternal short axis view. Abbreviations: $LVID_{ED}$, left ventricular end diastolic diameter; $LVID_{ES}$, left ventricular end systolic diameter; IVS, interventricular septal thickness; PWT, posterior wall thickness.
B. Parasternal long axis view demonstrating the LV end diastolic and end systolic volumes. The ejection fraction is attained using the formula:

$$\text{LV Ejection Fraction} = \frac{\text{LV End Diastolic Volume - LV End Systolic Volume}}{\text{LV End Diastolic Volume}} \times 100\%$$

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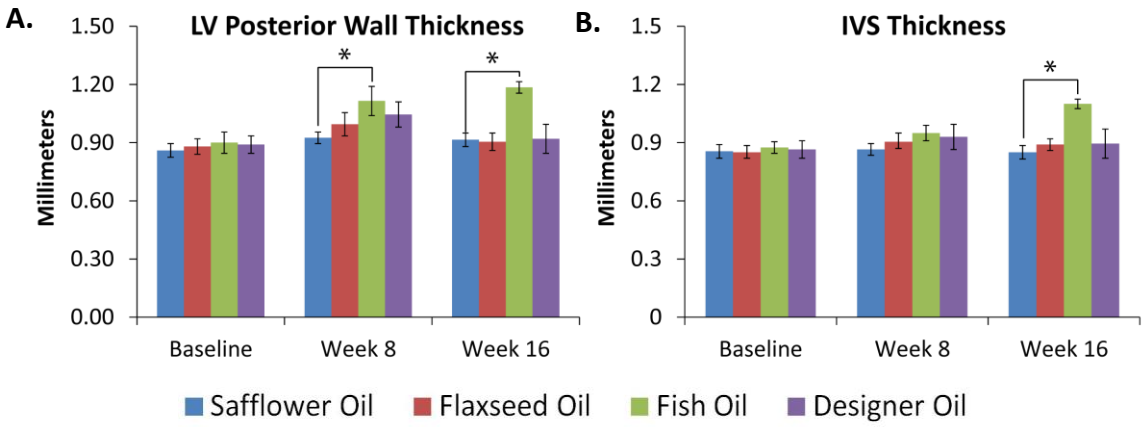

Figure 3. Left ventricular hypertrophy can be demonstrated by measurement of increased (A) left ventricular (LV) wall thickness and (B) interventricular septum thickness (IVS) in the ApoE^{KO} mice treated with fish oil. (n=10 per group; * p<0.05 treatment vs. control group)

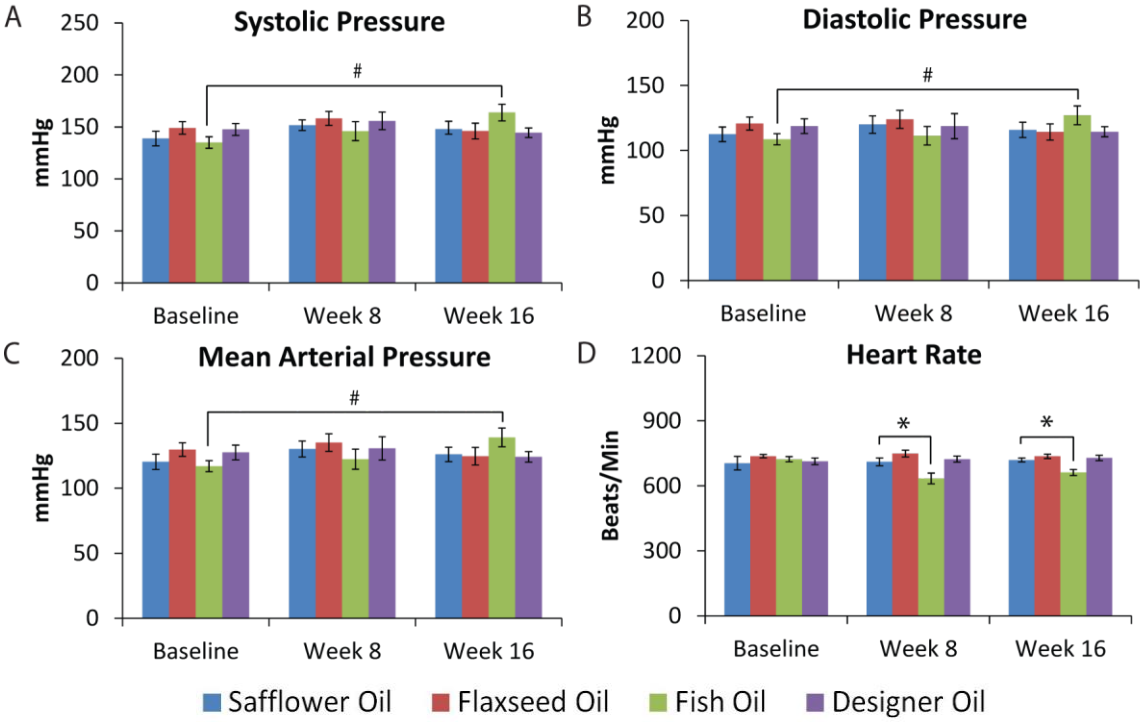

Figure 4. (A) Systolic blood pressure (SBP), (B) Diastolic blood pressure (DBP), (C) Mean arterial pressure (MAP) and (D) Heart rate (HR) were measured using the tailcuff method. No inter-group differences were seen between groups at baseline, week 8 or week 16. The fish oil treated group showed significant increase in SBP, DBP and MAP between baseline and week 16. (n=10 per group; # p<0.05 time-point vs. baseline; * p<0.05 treatment vs. control group)

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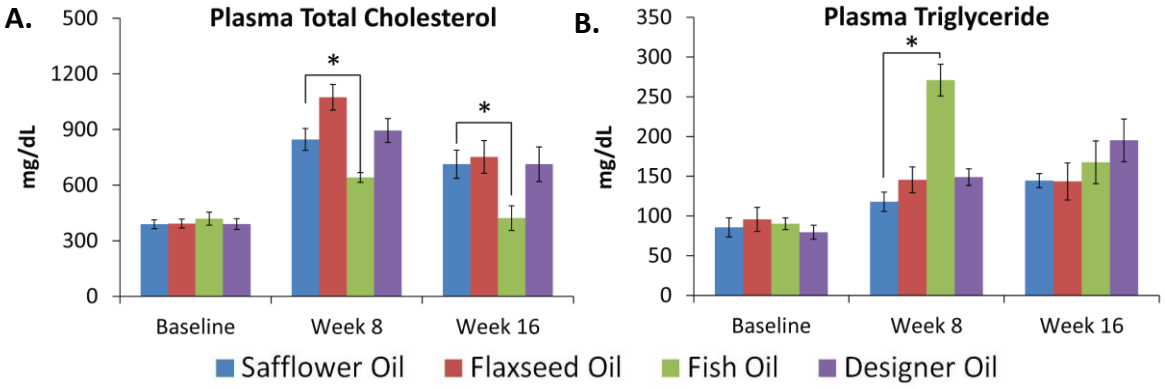

Figure 5. (A) Plasma total cholesterol (TC) levels increased in all treatment groups over the course of 16 weeks ($p<0.05$). Fish oil treatment reduced TC compared to control group ($p<0.05$). (n=10 per group; * $p<0.05$ treatment vs. control)
(B) Plasma triglyceride (TG) levels increased for fish oil treated groups relative to the control group at 8 weeks ($p<0.05$), but not at 16 weeks. (n=10 per group; * $p<0.05$ treatment vs. control)

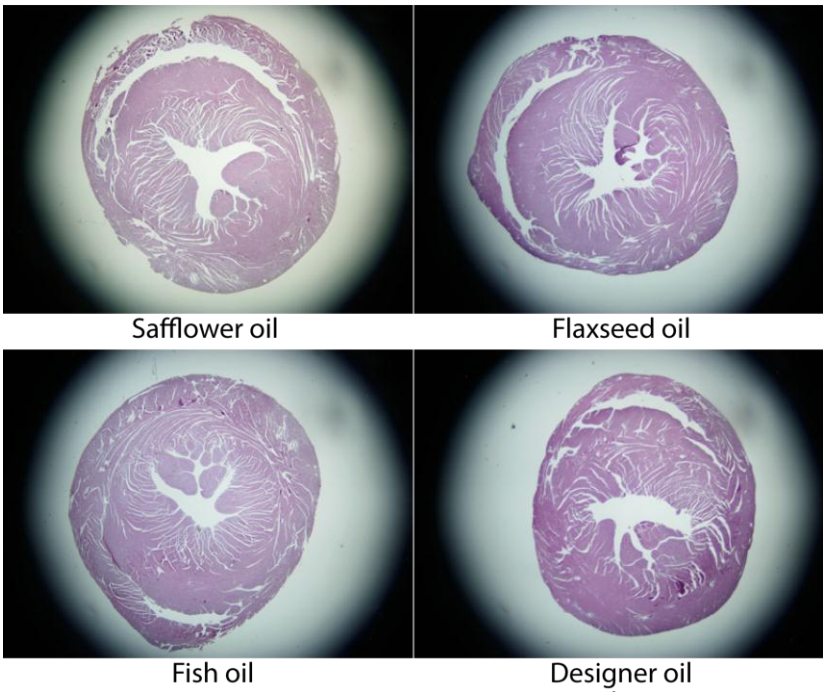

Figure 6. Microscope assessment (H&E stain, 20x magnification) of excised hearts after 16 weeks of high fat Western diet supplemented with the control and the treatment oils (safflower oil, flaxseed oil, designer oil and fish oil). No statistically significant hypertrophy is noted in treatment groups. (n=5 per group)

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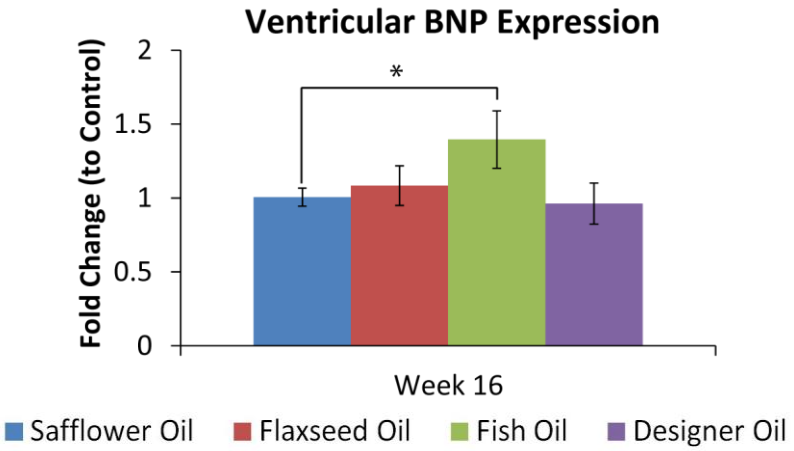

Figure 7. Ventricular brain natriuretic peptide (BNP) mRNA expression at week 16 was assessed using real time quantitative reverse transcriptase polymerase chain reaction (real time qRT-PCR). Relative quantification method was used to analyze the results, with the mean BNP expression of the control group set to 1. The BNP expression of treatment groups was measured relative to the control group. The fish oil treated group showed significant increase in BNP expression relative to the control. (n=10 per group; * $p<0.05$ treatment vs. control)

Table 1. Constitution of high fat murine chow supplemented with omega-3 fatty acids

	Safflower Oil	**Flaxseed Oil**	**Fish Oil**	**Designer Oil**
Energy (Kcal/g)	5.0	5.0	5.0	5.0
Fat (%)	42.0	42.0	42.0	42.0
Carbohydrates (%)	42.7	42.7	42.7	42.7
Protein (%)	14.5	14.5	14.5	14.5
Polyunsaturated Fatty Acid Composition				
n-3 Fatty acid	3.5%	57%	69%	33%
EPA	0.3%	Trace	34%	3%
DHA	0.2%	Trace	23%	2.3%
ALA & others	3 %	57%	12%	27.7%
n-6 Fatty acid	96.5%	43%	31%	67%
n-6: n-3 ratio	20	0.75	0.45	2.0