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Lipoxin-A4 in the rabbit model of atherosclerosis and liver steatosis

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Thesis

LIPOXIN-A4 IN THE RABBIT MODEL OF ATHEROSCLEROSIS AND LIVER STEATOSIS

by

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DEDICATION

I would like to dedicate this work to my supportive parents: you taught me to persist no matter what adversity I face. To my grandparents, who taught me the importance of patience and understanding in everything that I do. To my professors at Boston University School of Medicine. Dr. Moore, my compass in my graduate education, thank you for always pushing me to do better. Dr.Deeney, thank you for always being there to support me, no matter what questions or struggles I came to you with. Dr.Hamilton, thank you for taking a chance on me, and teaching me to be ferocious and relentless in the pursuit of excellence in science.

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LIPOXIN-A4 IN THE RABBIT MODEL OF ATHEROSCLEROSIS AND LIVER STEATOSIS

JASKAMAL SINGH

ABSTRACT

Background

Obesity is a global health problem that is associated with wide range of diseases, including atherosclerosis and Nonalcoholic fatty liver (NAFL) disease. Hepatic inflammation can cause cirrhosis, hepatic decompensation (liver failure) and cancer. Recent research now looks at the chronic systemic effects and inter-organ communication between atherosclerosis potentially promoting the development of NAFL. The resolution of inflammation is regulated naturally in the body by specialized pro-resolving mediators (SPMs). Immunoresolvents like ω6-derived Lipoxin A4 are suggested as a therapeutic strategy to overcome chronic inflammation and disease. In this study we investigated the therapeutic potential of Lipoxin A4 (LXA4) in cholesterol fed rabbit model of hypercholesterolemia, with atherosclerotic plaques and confined vascular endothelial injury and its effect on the progression of NAFL.

Objective

This is a continuation of studies pioneered in the Hamilton lab and an extension of the recent study by Taylor et. al in 2018[11] linking aortic plaque and liver disease. We will

now investigate the therapeutic potential of Lipoxin A4 on lipid-rich atherosclerotic plaques in cholesterol fed rabbits and its effect on the progression of NAFL to NASH.

Methods

In vivo magnetic resonance imaging (MRI) measured aortic atherosclerotic inflammation (with plaque Gd-enhancement), plaque size (vessel wall area), and composition, within rabbits fed normal chow or a 1% cholesterol-enriched diet. Biomarkers in the blood were monitored in the rabbits, with follow-up by histology, which included Masson's trichrome staining. Light Microscopy was used for liver imaging. Ex vivo MRI, T1W imaging was used to quantify VWA (vessel wall area), with Image J programming.

Results

Cholesterol-fed rabbits with and without aortic injury developed hypercholesterolemia, NAFL, and atherosclerotic plaques in the aorta. Elevated plasma gamma-glutamyl transferase (GGT; $p = 0.014$) and the ratio of liver enzymes aspartate and alanine aminotransferases (AST/ALT; $p = 0.033$) confirmed the progression of steatosis to non-alcoholic steatohepatitis (NASH). Histological images showed less fibrosis in those rabbits fed 1% CHOL diet with injury treated with LipoxinA4, when compared to 1% CHOL diet and injury alone. The plasma biomarkers showed a decrease in cholesterol (79%) and triglycerides (49.9%) in those rabbits given LXA4 therapy. The LXA4 treated 1% CHOL diet with injury group showed a marked decrease in the aorta vessel wall area

when compared to the 1% CHOL diet with injury, without treatment; as seen in ex vivo, MRI T1W imaging.

Conclusion

Lipoxin implementation in cholesterol fed rabbits that have localized regions of highly inflamed aortic atherosclerotic plaques, may contribute to the attenuation on the progression of NAFL to NASH as seen in histology and plasma biomarkers including; cholesterol and triglycerides. Lipoxin as a therapeutic has an effect on treating atherosclerotic plaques and attenuating atherosclerosis progression.

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LIST OF ABBREVIATIONS

AP-1……..…..activator protein-1

ANOVA……..Analysis of variance

CHOL..……..……Cholesterol

CVD……Cardiovascular disease

DW-MRI……Diffusion-weighted magnetic resonance imaging

GD….……Gadolinium

GPCRs……..……G protein-coupled receptor

HDL..……..…Lipoprotein

IL-8…….……..……interleukin 8

LDL…….……low density lipoprotein

LXA4……. Lipoxin A4

MRI…….magnetic resonance imaging

NAFL……..……non-alcoholic fatty liver

NASH ……non-alcoholic steatohepatitis

NRF2……..…….nuclear factor like 2

ONOO……peroxonitrite anion

PI3K……phosphatidylinositol-4,5-bisphosphate 3-kinase

PLM…….……Polarized Light Microscopy

RVV-X……..…Russell's Viper Venom

SAA……..serum amyloid A

SPMs……..……Specialized pro-resolving mediators

T1W……..…T1-weighted

T2W…….…… ..T2-weighted

VLDV…… very-low density lipoprotein

VWA …….……vessel wall area

INTRODUCTION

Non-Alcoholic Fatty Liver Disease and Steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is found in patients who are not considered alcoholic; it causes liver damage.[24] NAFLD made up of a spectrum of stages from simple steatosis to inflammation associated fibrosis and finally cirrhosis.[24] Steatosis is the infiltration of liver cells with fat, associated with disturbance of metabolism.[26] Non-Alcoholic Steatohepatitis (NASH) develops in patients with at least one of the following risk factors: obesity, dyslipidemia, and glucose intolerance.[26] The advancement of steatosis in NAFL leads to NASH; it is often seen together with fibrosis and, at some point, becomes irreversible with subsequent progressing to cirrhosis.[27] Fibrosis is the encapsulation or replacement of injured tissue by a collagenous scar. Liver fibrosis results from the perpetuation of the normal wound healing response, which results in an abnormal development of prolonged fibrogenesis (connective tissue production and deposition). Cirrhosis is an advanced stage of liver fibrosis that includes alteration of the hepatic vasculature.[28]

Steatosis can result from hepatic triglyceride accumulation. Possible mechanisms for steatosis include decreased oxidation of fatty acids and increased free fatty acids being delivered to the liver, which can cause reduced synthesis of very low density lipoprotein (VLDL) and increased hepatic triglyceride synthesis.[29] Inflammation may result from lipid peroxidative damage to cell membranes. These changes can stimulate hepatic stellate cells, resulting in fibrosis.[29]

The accumulation of hepatic fat is associated with hepatic inflammation, which increases the risk of liver disease; cirrhosis, hepatic decompensation (liver failure) and/or hepatocelluar carcinoma.[24] The prevalence of NAFL in the global population is around 25% and in the presence of obesity, as high as 51%.[25]

Rabbit Model of Atherosclerosis

Rabbit studies have been used to discover novel aspects of cardiovascular disease relevant to humans, including the discovery of the role of nitric oxide and the role of vascular inflammation.[22] Cholesterol-fed rabbits can develop plaques that exhibit similarities to human coronary and carotid plaques, which makes them useful as a pre-clinical model.[2] The rabbit model we used, replicates many features of human cardiovascular disease, including thrombosis as seen in the human arteries.[18]

Our model applies pharmacological triggering to test the vulnerability of advanced plaque disruption and thrombosis with Russell viper venom and histamine, which combines activation of coagulation factor Xa and Va and vasoconstriction with histamine to stimulate an acute event that can lead to myocardial infarction.[18] The development of vulnerable plaques can be sped up with the introduction of balloon de-endothelialization.[18] An advantage of the rabbit model is that thrombosis can be experimentally controlled, which allows for the study of aortic plaques with noninvasive in-vivo MRI and other invasive methods like ex-vivo MRI done after aortic excision.[18] Plaque rupture is characterized by a necrotic core with a overlying highly inflamed thin fibrous cap infiltrated by macrophages. The vessel wall is also highly inflamed and has

monocyte/neutrophil/macrophage/leukocyte infiltration.[23] A recent study showed that oral inflammation with Porphyromonas gingivalis exposure exacerbated aortic atherosclerosis and systemic inflammation in cholesterol fed rabbits.[21] More recently Taylor et. al discovered that cholesterol-fed rabbits with induced vascular endothelial injury had more inflamed aortic plaques and NAFL with fibrosis, suggesting that the advanced aortic plaque inflammation had a systemic effect.11

Lipoxin and Resolution of Inflammation

The role of inflammation and some of the underlying mechanisms that contribute to atherogenesis have been illuminated by recent studies. An exciting new area of focus with broad implications is the linkage inflammation with other chronic diseases, which is stimulating greater recognition of nutrition and natural mechanisms of homeostasis. A prolonged nutrient excess of some nutrients results in chronic activation of the immune response and associated inflammation.[1]

A therapeutic strategy that has the potential overcome chronic inflammation and disease is the use of naturally produced specialized pro-resolving mediators (SPMs). Cellular and plasma levels of SPMs vary, and chronically low levels of SPMs may predispose the progression of chronic vascular inflammation and vulnerability to coronary atherosclerosis and thrombosis.[14] An example of one such molecule is lipoxin which is secreted by immune cells such as neutrophils and macrophages.[6] Lipoxins are products of the arachidonic acid pathway, and can be stimulated by aspirin treatment as described by Serhan et al.[7] Endogenous biosynthesis of LXA4 (Lipoxin A4) occurs through the interaction of leukocytes with epithelium, endothelium or platelets.[7] Lipoxins

act at specific GPCRs (G protein-coupled receptor) as agonists to regulate cellular responses related to inflammation and resolution.[7] Lipoxin binds to GPCR ALX/Formyl peptidyl receptor; which prevents binding of other pro-inflammatory ligands like SAA (serum amyloid A) and that triggers a wide range of cytoplasmic signaling cascades, as schematically illustrated by Chandrasekharan et al, in Figure 1.[8] Lipoxins resolve inflammation by activating the PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) and AKT pathways in macrophages, which increases the life span of the macrophage.[8] Lipoxin controls synthesis and release of pro-inflammatory cytokines by increasing the mRNA level of suppressors of cytokine signaling and preventing the transcription of inflammatory cytokines like IL-8 by inhibiting NFκB and AP-1.[8]

Neutrophils are a key part of the innate immune response, and lipoxins stimulate migration of neutrophils to the site of inflammation by increasing their cytosolic calcium (Ca2+) levels.[9] The increase in calcium promotes cytoskeleton assembly which aids in extension of neutrophil pseudopods, making transendothelial migration easier.[10] Macrophages are recruited to the site of infection after neutrophils.[8] Lipoxins also promote resolution of inflammation by delaying the apoptosis of macrophages.[8] Lipoxins stimulate activation of the PI3K/Akt and ERK/nuclear factor like 2 (Nrf2) pathways that have a role in the inhibition of apoptosis in macrophages.[12] T-cell secreted cytokines, such as tumor necrosis factor alpha (TNFα) which promotes inflammation can be inhibited by lipoxin. [13]

Lipoxins also help prevent tissue injury by inhibiting the formation of peroxonitrite anion (ONOO) at the site of inflammation.[16] Peroxonitrite interacts with

lipids, DNA, and proteins through direct oxidative reactions or indirect, radical-mediated mechanisms. [15] These reactions trigger cellular responses that can be subtle variations of cell signaling to damaging oxidative injury, committing cells to necrosis or apoptosis. [15]

Lipoxin also attenuates the accumulation of nuclear factor κB (NFκB) and activator protein-1 (AP-1) in the nucleus, which consequently reduces IL-8 production. Inhibiting IL-8 secretion also reduces neutrophil accumulation and activation.[17] Therefore, specialized pro-resolving mediators like lipoxins may provide new possibilities to design novel "resolution-targeted" therapy, which could effectively control inflammation.

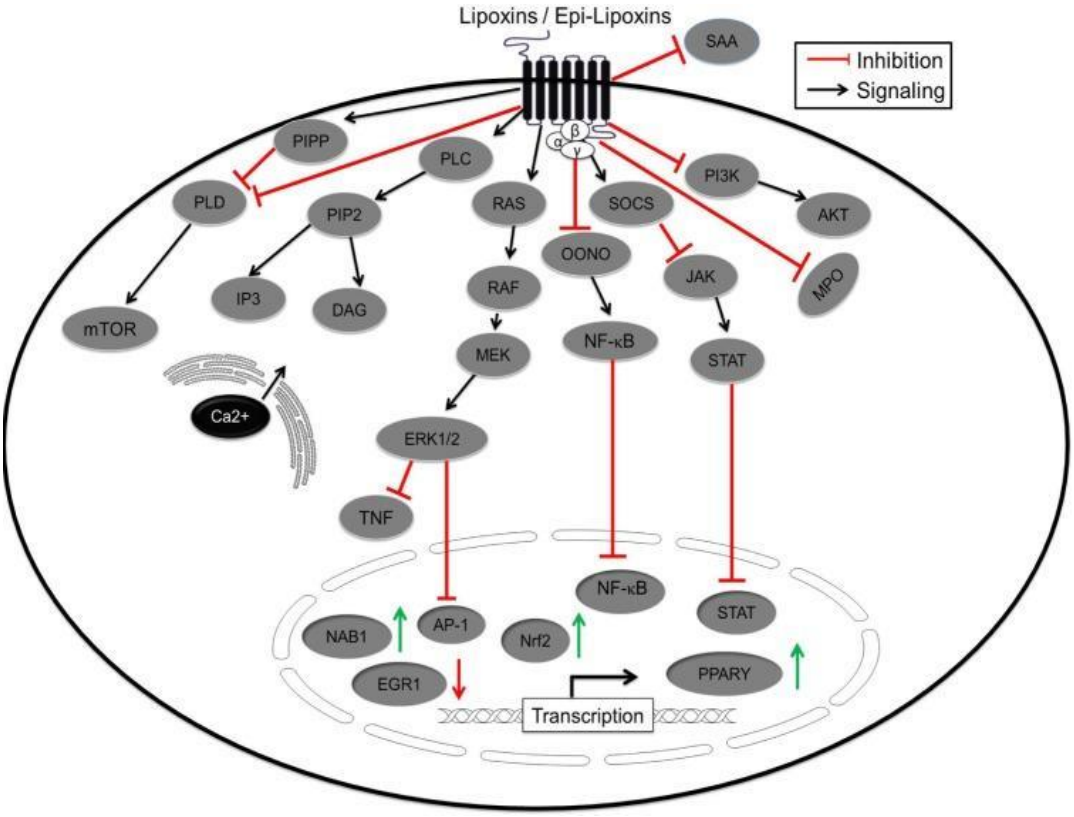

Figure 2. The Effect of Lipoxins on various transcription factors and cytoplasmic signalling cascades. (Lipoxins: nature's way to resolve inflammation, Chandrasekharan et.al)[8]: Lipoxin modulates various transcription factors such as nuclear factor κB, activator protein-1, nerve growth factor-regulated factor 1A binding protein 1, and peroxisome proliferator activated receptor γ and control the expression of many inflammatory genes.[8]

Abbreviations: AP-1, activator protein-1; DAG, diacyl-glycerol; EGR1, early growth response 1 gene; ERK, extracellular signal-regulated kinase; IL-8, interleukin 8; IP3, inositol triphosphate; JAK, Janus kinase; MEK, mitogen-activated protein kinase kinase; MPO, myeloperoxidase; mTOR, mammalian target of rapamycin; NAB1, NGFIA binding protein 1; NFκB, Nuclear factor κB; Nrf2, nuclear factor like 2; ONOO, peroxonitrite anion; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PLD, phospholipase D; PPAR, peroxisome proliferator activated receptor; SAA, serum amyloid A; TNFα, tumor necrosis factor alpha; SOCS, suppressors of cytokine signaling; PIPP, polyisoprenyl phosphate.

METHODS

Rabbit model of atherosclerosis

The methodology in this thesis work is a continuation of the study done by Taylor et. al in 2018.[11] Female and male New Zealand White rabbits were fed normal chow and a 1.0% CHOL containing chow diet for a course of three months (**Table 1** includes all of the study groups and abbreviations). One of the study groups was 1% CHOL + injury; N=4. In this group we induced atherosclerotic lesions with the 1% CHOL diet (TestDiet, Saint Louis, MO) in combination with endothelial cell injury via a balloon catheter procedure during general anesthesia (acepromazine, 0.75 mg/kg; ketamine, 35 mg/kg; xylazine, 2.5 mg/kg for each rabbit).[11] The second group (N=3) was treated with LXA4 (8 micrograms 3X per week, for a period of 4 weeks) . A Control group (Normal diet) was fed a normal chow and not subjected to balloon injury (N=3; female). The normal diet consisted of a chow with 16.5% protein, 41% carbohydrates, 22.5% fiber, 2% fat. The 1% CHOL diet consisted of a chow that included 10,000 ppm cholesterol, 14.4% protein, 42.8% carbohydrates, 23.7% fiber, 2.4% fat by weight.[11] The remaining mass consisted of vitamins, minerals, ash and moisture.[11]

We induced plaque formation in the aorta and tested whether it formed a luminal thrombus in both 1%CHOL fed groups with injury.[11] We used the standard pharmacological procedure that from our past studies of the rabbit model of human atherosclerosis [3,4,] At the end of the three months, intraperitoneal injections (two times, separated by 48 hours) of coagulation cascade factor-X activating enzyme isolated from Russell's Viper Venom (RVV-X 0.15 mg/kg IP; Enzyme Research, South Bend, IN)

followed by histamine injection (0.02 mg/kg IV; Sigma Aldrich, St. Louis, Mo) after 30 min were used to trigger rupture of vulnerable plaques.[11]

At the end of the 2 months, we administered a dosage of Lipoxin orally 8 micrograms 3X per week, for a period of 4 weeks to the 1% CHOL + Injury + LXA4 group. Timeline of the procedure included 8 weeks of 1% CHOL feeding in the CHOL-fed experimental groups for 8 weeks, followed by 4 weeks of normal chow feeding. For the LXA4 group, the treatment was given for a period of 4 weeks between week 8 and week 12 (can be seen in **Figure 2).**

Serial magnetic resonance imaging (MRI) of aortic plaque

Assessment of the aorta in each rabbit for atherosclerosis was done by in vivo MRI using a Philips Achieva 3T scanner with a 16 channel TR knee coil under general anesthesia and free breathing conditions.[11] The MRI protocol included T1-weighted (T1W) and T2-weighted (T2W) imaging substituted with non-gated 3D black-blood sequences repeated at the end of the second and third months.[11]

In excised aortas high field MRI (11.7T; Bruker Biospin Corporation, Billerica, MA) was used to study sliced aortic segments that were rinsed in cold saline, fixed in 10% neutral buffered formalin, and then placed in MR-signal inert fomblin oil (Sigma Aldrich).[11] The fomblin oil suppresses background signal, providing high signal-to-noise proportion during visualization of the tissue.[11] Aorta segments were stored in an ice-water bath and were equilibrated to room temperature before the ex vivo MRI protocol.[11] All experiments were conducted with high resolution T1W imaging (28 μm2 in plane

resolution with 500 μm slice thickness).[11] Diffusion-weighted MRI (DW-MRI; 39 μm2 in plane resolution with 500 μm slice thickness with a diffusion-weighting B value of 3,000 s/mm2). The diffusion-weighting B value was selected and optimized based on the protocol developed by Qiao et al., for ex vivo visualization of cholesteryl esters in human and rabbit plaques.[18,19,11]

Thrombus detection

At the end of the three-month experiment (Figure 1), thrombus formation was measured in ImageJ (NIH, Bethesda, MD) by comparing of matched corresponding regions in the MR images (pre- and post-contrast T1W images) obtained before and after triggering.[11]

Plasma biomarkers

Prior to MRI scans, blood was collected (total volumes per draw of 2-4 ml) in heparin blood collection tubes for quantification of liver enzymes and lipids (mg/dL). [11] The biomarker analysis included measurements of the liver enzymes including; alanine aminotransferase (AST), aspartate aminotransferase (ALT), and gammaglutamyl transferase (GGT). The ratio of AST and ALT, is called the De Ritis ratio, was used to assess liver damage in NASH.[20] Other biomarkers that we assessed were total plasma triglycerides and plasma free CHOL. [11] All blood biomarkers were measured by the Abbott Piccolo Xpress Analyzer (Abbott, Chicago, IL).[11]

Histology

The liver was fixed with formalin and paraffin embedded. The liver sections were stained with Masson's Trichrome.[11] Images of the liver post mortem were taken. Masson's trichrome revealed fibrotic areas that stained blue, these areas were quantified

after microscopic imaging and in multiple fields of view at the same magnification with ImageJ software using color threshold to quantify liver fibrosis.[11] Masson's trichrome stain is the most common histological method applied to the liver. The stain imparts a blue color to collagen against a red background of hepatocytes and other structure. It stains type 1 collagen, that is present in portal tract and vessel walls. It highlights the presence and distribution of fibrosis as a result of liver injury. It is used for classifying stages of liver disease, and helps to delineate patterns of injury, such as the perisinusoidal fibrosis associated with steatohepatitis.[30]

Statistical analysis

Statistical comparison was carried out on data derived from 1% CHOL + injury, 1% CHOL + injury + LXA4, and normal diet rabbit groups. In addition to comparisons between groups at study time points, baseline and longitudinal measurements were included where possible.[11] Two-sample student's t-test for the mean difference per rabbit, one-sided or one sample t-test for comparison with baseline reading was carried out in Excel (Microsoft Corporation, Redmond,WA).[11]

Table 1. The rabbit groups used in this study.

Groups used in this study	**Abbreviation**
Normal diet + no injury	Normal diet
1% cholesterol diet + endothelial injury	1% CHOL + injury
1% cholesterol diet + endothelial injury + Lipoxin A4	1% CHOL + injury + LXA4

*Abbreviation denotes the abbreviated names used in this study

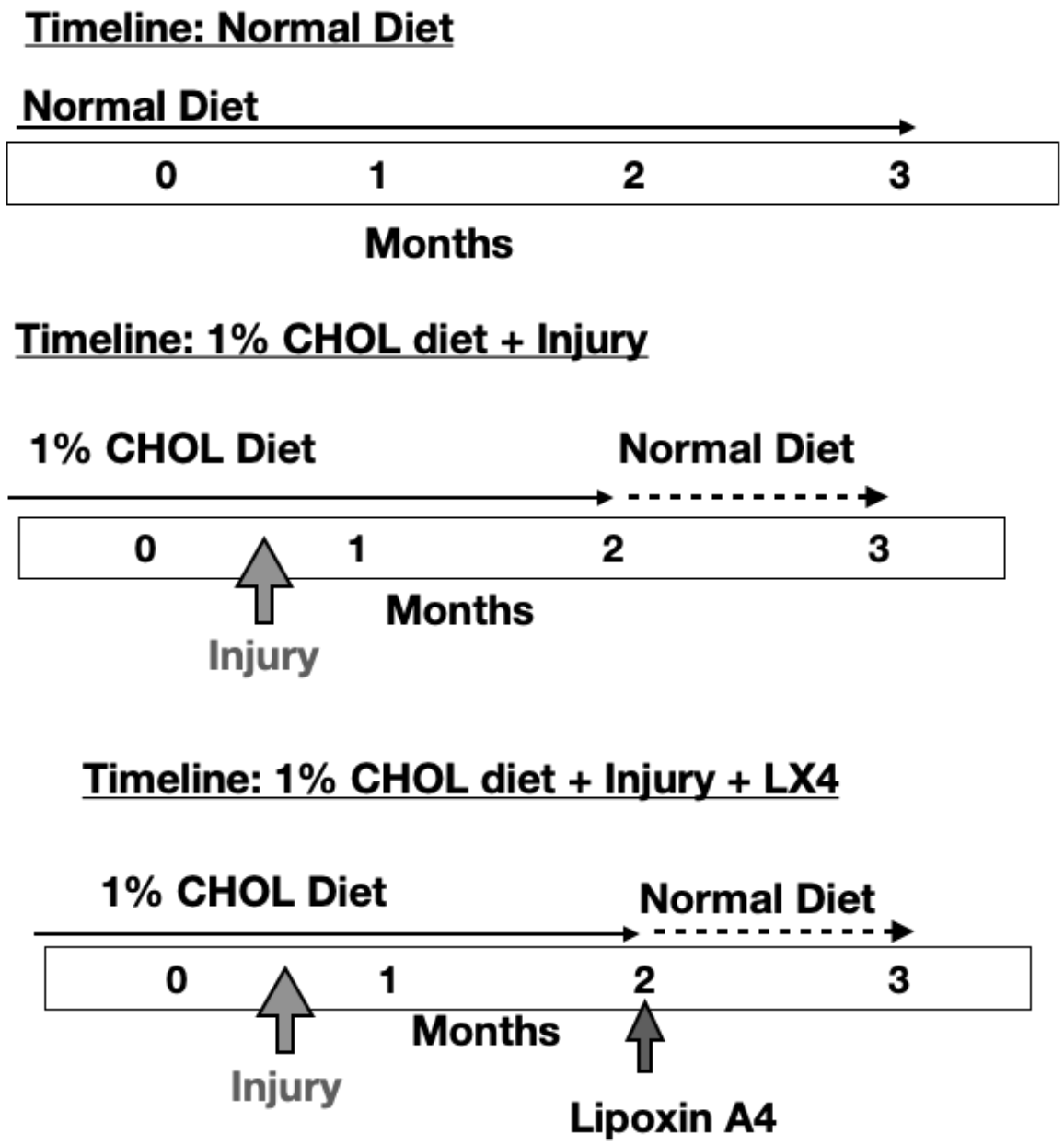

Figure 2: Timeline of Protocol, Including feeding, injury and treatment in all 3 groups.

RESULTS

Plasma Biomarkers:

Liver enzyme levels of ALT, AST, and GGT confirmed the presence of NAFL. A significant increase in liver enzymes from baseline levels (Table 2; Change in liver enzymes in plasma with 1% CHOL diet and endothelial injury) was observed in the blood of the CHOL-fed + injury rabbits. Baseline readings are defined as blood plasma samples obtained prior to initiation of 1% CHOL diet. Readings in blood were also obtained at three months after pharmacologic triggering. GGT, AST, and ALT were all increased by 164%, 356%, and 158%. The ratio of AST to ALT increased from 0.63 to 1.11; a 176% increase and this effect is substantial.

At the end of the second month of the preparation protocol, the 1% CHOL diet was switched to normal diet, as in our standard procedure, and LXA4 was initiated in the therapy group. The blood cholesterol and triglycerides were measured at 2 month and 3 months. At the 2-month point, (prior to any therapy) both groups that were given the 1% CHOL diet and injury had high cholesterol and triglyceride levels (812.8, 62 respectively in 1% CHOL diet + injury) and (2065, 82.3 respectively in CHOL diet + injury + LXA4). At the beginning of the 3rd month, the rabbits were all placed on a normal diet. After 3 months, when the therapy group had undergone lipoxin A4 treatment, the triglycerides and cholesterol were measured and compared. After switching to a normal diet, at the end of 3 months, the without therapy group (1% CHOL diet+ injury) had a 39.5% decrease in cholesterol and had a increase in triglycerides. The therapy group (1% CHOL diet+

injury+ LXA4) showed a 79.8% decrease in cholesterol and 49.9% decrease in triglyceride from the implementation of lipoxin treatment. There were much higher lipids in the therapy group before the treatment, this was most likely due to the cholesterol diet.

Liver Enzyme Biomarker	Baseline	After 1% CHOL + injury	P-value
GGT (U/L)	6.3 ± 0.5	10.3 ± 1.3	0.014
AST/ALT ratio	0.63 ± 0.12	1.11 ± 0.18	0.033

Table 2: Change in liver enzymes in plasma with 1% CHOL diet and endothelial injury (N=4). Readings from blood plasma were compared in 1% CHOL + injury rabbits to baseline readings. Baseline readings are defined as blood plasma samples obtained prior to initiation of 1% CHOL diet. Readings in blood were also obtained at three months after pharmacologic triggering. The P-value displayed was obtained from the student's t-test comparing the baseline and after trigger readings.

	No Therapy: 1% CHOL diet+ injury (N=4)			**Therapy:** 1% CHOL diet + injury + LXA4 (N=3)		
	2 months	3 months	% decrease	2 months	3 months	% decrease
Cholesterol	812.8	467.5	39.6%*	2065.0	485.7	79.8% **,†
Triglyceride	62.0	89.7	increase	82.3	43.0	49.9%**

Table 3 Blood lipids before and after four weeks of LXA4 therapy. The * indicates a p-value is less than 0.05 and ** p-value less than 0.005 using a one-sided t-test comparing two- and three-months blood draws from the same group. † indicates p-value less than 0.05 by two-sided t-test when comparing the no therapy and therapy groups.

Histology

Masson's trichrome staining of the liver and whole liver images

Masson's trichrome stain imparts a blue color to collagen against a red background of hepatocytes and other morphological structures. It stains type 1 collagen blue, which is present in portal tract and vessel walls. As shown in Figure 3 the normal diet rabbit shows normal distribution of collagen fibers, stained dark purple, around a portal tract. 1% CHOL+ injury shows a marked increase in blue staining, showing an increase in collagen fibers around the blood vessels in the portal tract. 1% CHOL+ injury + LXA4 shows much less blue uptake in the liver, showing a lower distribution of collagen fibers around a portal tract, when compared to the cholesterol + injury group with no treatment. Whole liver photographic images (Figure 3) taken post mortem dramatically illustrate liver disease and effective therapy by LXA4. The normal diet rabbit liver is lean with good hepatic circulation and a bright red color. With CHOL feeding, color changed to a paler pink color, meaning decreased hepatic circulation. In addition, the rabbit liver of the 1% CHOL + Injury + LXA4 is a pale color, however has less color loss when compared to the no treatment rabbit (1% CHOL + injury). See **figure 3** for complete details.

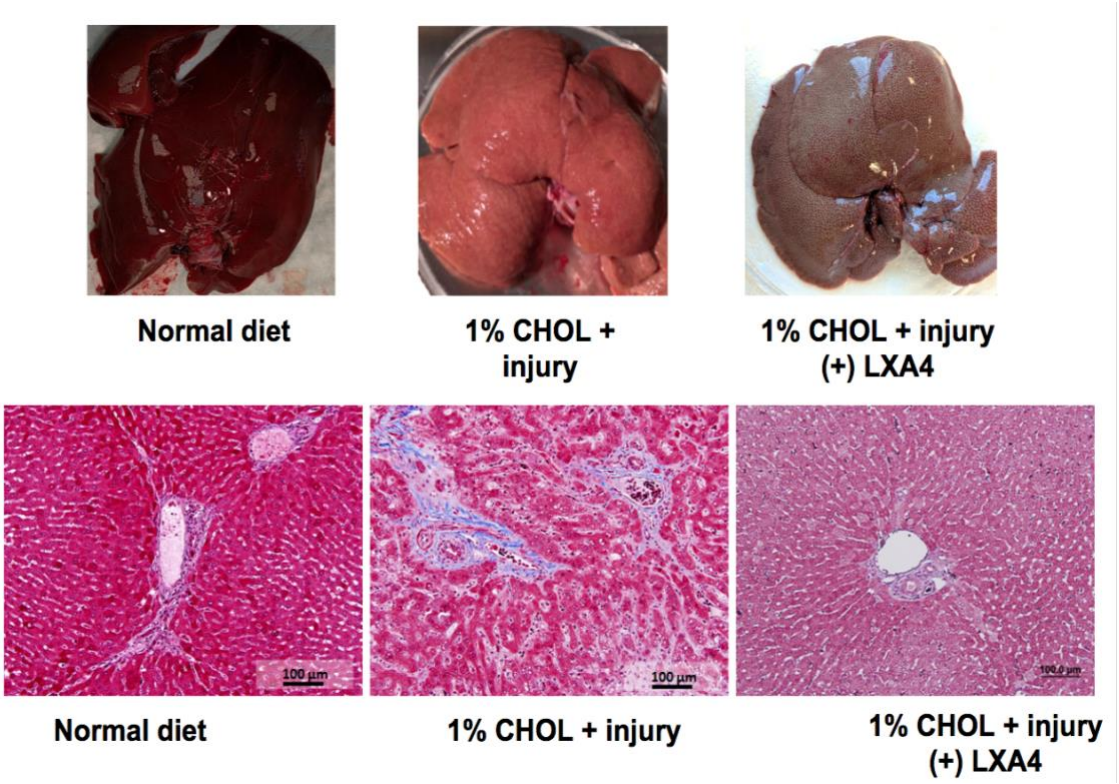

Figure 3 Trichrome Staining Microscope Liver and Whole Liver Images
Masson's Trichrome staining of collagen within the liver tissue. Normal diet shows normal distribution of collagen fibers, stained dark purple, around a portal tract. 1% CHOL+ injury shows marked increase in blue staining, therefore an increase in collagen fibers around the blood vessels in the portal tract. 1% CHOL+ injury + LXA4 shows less blue uptake in the liver, so a lower distribution of collagen fibers around a portal tract. The whole liver images are taken post mortem; Normal diet rabbit liver is lean with bright red color. 1% CHOL + injury rabbit liver shows lots of fibrosis and decreased hepatic circulation, is a paler pink color. 1% CHOL + Injury + LXA4 has some fibrosis and is less pale and has less much fibrosis than the no treatment rabbit (1% CHOL + injury).

Quantification of Liver Fibrosis

The quantification of trichrome staining revealed how much of the liver area (%) took up masson's trichrome, with the color blue reflecting the amount of collagen in the liver. Those areas with greater collagen meant greater fibrosis (Figure 3). Figure 4 shows a bar graph quantification. The 1% CHOL diet + injury group had the most trichrome staining (~9%), and the normal diet group had the least amount of trichrome staining (<2%). The 1% CHOL+ injury+ Lipoxin A4 therapy group showed a marked decrease when compared to the experimental group with no treatment, with ~3% staining of the liver area.

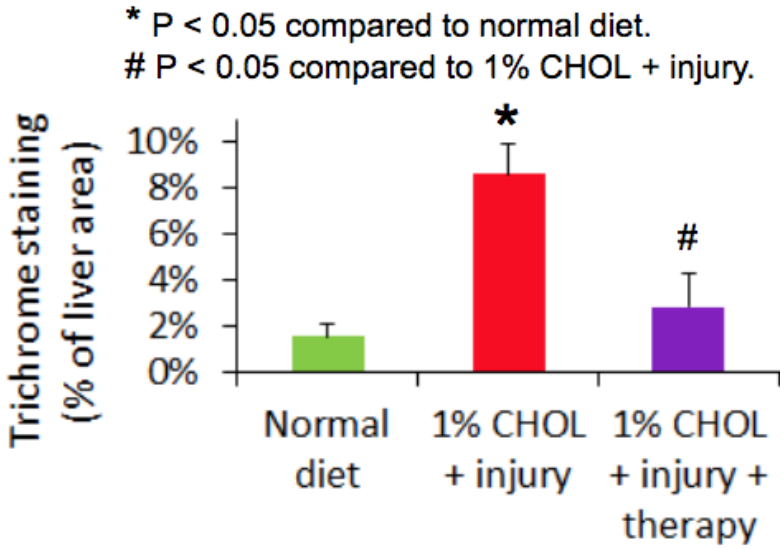

Figure 4 Quantification of Liver Fibrosis. Trichrome staining revealed how much of masson's trichrome was taken up in the liver; areas that took up more reflected higher collagen and fibrosis in the liver. Those areas with greater collagen meant greater trichrome staining. 1% CHOL diet + injury group had the most trichrome staining (*p <0.05) compared to normal diet). The normal diet group had the least amount of trichrome staining. The 1% CHOL+ injury+ Lipoxin A4 therapy group showed a marked decrease when compared to the 1% CHOL+ injury group with no treatment (#p <0.05 compared to 1% CHOL + injury).

Aorta vessel wall area (mm²)

The normal diet group had an overall smaller aorta vessel wall area than injury and Lipoxin treated groups. 1% CHOL diet + injury group had the highest aorta vessel wall area. The LXA4 treated groups had lower aorta vessel wall areas when compared to the 1% CHOL + injury with no treatment. The 1% CHOL diet + injury group had an overall larger aorta vessel wall area (mm.) than normal group (*p<0.05 compared to normal). The LXA4 treated group had a lower aorta vessel wall area when compared to the 1% CHOL + injury with no treatment (# p < 0.05 compared to injury).

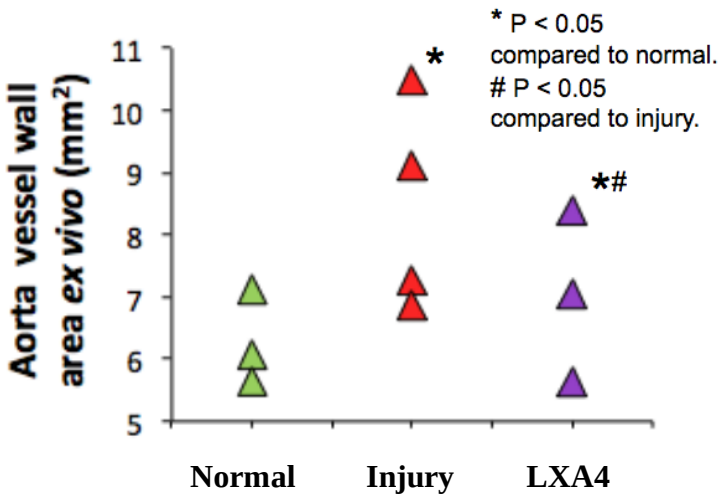

Figure 5 Aorta vessel wall area with treatment (ex vivo). Normal diet group is labelled as normal (N=3). 1% CHOL diet + injury group is labelled as injury (N=4). 1% CHOL diet + injury group is labelled as injury (N=4). 1% CHOL diet + injury + LXA4 group is labelled as LXA4 (N=3). The 1% CHOL diet + injury group had an overall larger aorta vessel wall area (mm^2) than normal group (*p <0.05 compared to normal). 1% CHOL diet + injury group had the highest aorta vessel wall area. The LXA4 treated groups had a lower aorta vessel wall area when compared to the 1% CHOL + injury with no treatment(# $p < 0.05$ compared to injury).

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DISCUSSION

The focus of this study was to investigate the therapeutic potential of Lipoxin A4 on the progression of fibrosis in NAFL, in a rabbit model of atherosclerosis. To confirm the presence of NAFL and effects of treatment we measured at liver enzymes in blood samples. We used the De Ritis[20] ratio to assess the liver damage,

As shown in our plasma biomarkers, cholesterol fed rabbits treated with LXA4 had a marked decrease in triglycerides and free blood cholesterol when compared to those cholesterol fed rabbits with no treatment. In a large clinical cohort study, diabetic patients with advanced fibrosis (n=204) had the mean AST/ALT ratio of 0.98, advanced fibrosis in this study was defined by histological bridging fibrosis or cirrhosis.[31] We also measured GGT, which also significantly increased after the 1% CHOL diet and injury. GGT is a measure of liver function because it is responsible for the extracellular catabolism of the antioxidant glutathione and is suspected to be important in chronic inflammation and systemic oxidative demand.[11] In future directions of study, the GGT levels could be compared prior and post immunoresolvent treatment in the atherosclerosis rabbit model.

Rabbit vasculature is similar to humans, and the diameter of the aorta is similar that to average diameter of human coronaries, making the rabbit model of atherosclerosis relevant for clinical applications. Rabbits in this study developed plaques similar to humans. In addition, blood cholesterol and triglycerides were elevated during cholesterol feeding and lowered after return to normal chow in the third month. The lipoxin therapy in 1% CHOL diet + injury + LXA4 resulted in a larger 79.8% decrease in cholesterol and

49.9% decrease in triglyceride. The treatment of Lipoxin indicates that the immuoresolvent can decrease free blood cholesterol and triglycerides. The decrease in these plasma biomarkers is clinically significant. In recent studies, the presence of hypercholesterolaemia was investigated; it was shown that hypercholesterolaemia lead to cholesterol accumulation in macrophages and other immune cells, which promotes inflammatory responses.[32] Therefore, a decrease in cholesterol and triglycerides systemically would lead to decreased systemic inflammation, and could attenuate the progression of a inflammatory pathological disease. SPM's like LXA4, shown in our study; lead to a decrease in triglycerides and cholesterol, which has the implication that when used as a therapeutic could lower overall systemic inflammation.

There was a marked change in the size of the aorta vessel wall area with induction of atherosclerosis with balloon injury and cholesterol feeding. In our study, lipoxin demonstrated a cardio-protective effect; the treatment of LXA4 decreased the size of aorta vessel wall area in those rabbits with plaques established from a cholesterol diet and vascular endothelial injury. Lipoxins attenuate processes associated with atheroscleortic plaque formation including; neutrophil recruitment, activation and neutrophil extracellular traps formation. Lipoxins also enhance resolution by enhancing efferocytosis.[36] From our past studies with a similar immunoresolvent (Resolvin E1), we showed that atherosclerosis can be attenuated from SPM therapeutic treatment effectively. SPM therapy is simpler and safer than implementing other invasive procedures.[32] A SPM therapeutic approach would likely have few side effects and provide long-term safety with respect to atherosclerosis and CVD.

The LXA4 therapy had a beneficial effect on the fibrosis of the liver. Our study showed that in treatment of LXA4 reduced fibrosis compared to livers of untreated rabbits. Masson's trichrome stain is a common histology special stain for liver and cardiac biopsies, they conveniently highlight the amount and distribution of fibrosis.[33] Because of background staining, trichrome stains also allow easy evaluation of liver architecture.[33] 1% CHOL+ injury shows marked increase in blue staining when compared to the normal diet group. The 1% CHOL + injury group showed an increase in collagen fibers around the blood vessels in the portal tract (**figure 3)**. 1% CHOL+ injury + LXA4 shows less blue stain uptake in the liver, so a lower distribution of collagen fibers around a portal tract. Those rabbits that were given the 1% CHOL and the endothelial injury had a significant amount of collagen fibers around the portal tract, indicating that there was severe fibrosis. The rabbits treated with LXA4 had less of fibrosis, although there was some blue staining, there was substantially less collagen deposition in the liver (see **figure 3**). From other studies, it has been shown that other SPMs (RvE1, Protectin D1, and D-series Rvs) attenuate obesity-induced liver disease.[34] Recent data has highlighted a protective effect of Lipoxins and other arachidonate-derived mediators in cardiovascular disease associated with increased reverse cholesterol transfer.[35] From our study, lipoxins attenuated the fibrosis of the liver; showing a portal tract that looked closer to normal than those cholesterol fed rabbits with injury and without treatment.

Collectively, the results from this study shows that LXA4 plays a role in modulating hepatic inflammation and fibrogenesis during NASH progression. LXA4 demonstrates the potential of using SPM's to attenuate obesity-induced pathologies.

The use of the rabbit model is advantageous for studying human disease instead of persons directly. Rabbits have served as a primary experimental model for some human infectious diseases, because of their susceptibility to infection and the similarity of pathogenesis to that in humans.[37] Rabbit vasculature is similar to humans, and the diameter of the aorta is similar that to average diameter of human coronaries, making the rabbit model of clinically ideal for cardiovascular research.[22] Studying the liver is challenging; studies are limited by lack of tissue except for liver biopsies, which are rarely performed and autopsies. The use of liver MRI is also less well developed than vascular MRI.[21] Studies correlating progression and regression are of liver disease is almost impossible.[37] The induction of liver disease in human for controlled experiments is unethical. There is a rapid development of rabbit genomics, proteomics, transgenic and knockout lines, and rabbit-specific reagents, the use of rabbits as experimental models in biomedical research will likely increase.[37] The rabbit model has the ability to bridge the gap between rodents and the large animal models often required for preclinical and translational research.[37]

The future directions for this study could include investigating the effects of LXA4 on immunological biomarkers like white blood cells, neutrophils, macrophages. Future studies of the pro-resolving effects of SPMs are to be investigated in other inflammatory pathologies including rheumatoid arthritis and psoriasis.

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